03-21-07

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Docket No. D0287 NP

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicants	:	Riccardo Attar, et al.
Application No.	:	10/620,514 Confirmation No. : 3956
Filed	:	July 16, 2003
For	:	TRANSGENIC NON-HUMAN MAMMALS EXPRESSING A REPORTER NUCLEIC ACID UNDER THE REGULATION OF ANDROGEN RESPONSE ELEMENTS
Group Art Unit	:	1632
Examiner	:	Joanne Hama

Princeton, New Jersey 08543
November 13, 2006

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Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

AMENDED APPEAL BRIEF

This Amended Appeal Brief is filed in response to the Notification of Non-Compliant Appeal Brief mailed February 27, 2007.

I. REAL PARTY IN INTEREST

The real party in interest is Bristol-Myers Squibb Company, a corporation organized and existing under the laws of Delaware and having an office and place of business at P.O. Box 4000, Princeton, NJ 08543. The present assignee of this application is Bristol-Myers Squibb Company. 37 C.F.R. § 41.37(c)(1)(i).

II. RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences known to Appellant or their legal representatives that will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal. 37 C.F.R. § 41.37(c)(1)(ii).

III. STATUS OF CLAIMS

Claims 1-13, 18, and 19, set forth in Appendix A, stand rejected and are on appeal. Claims 14-17 were previously canceled. Claim 20 was canceled in the amendment filed concurrently with this Appeal Brief. 37 C.F.R. § 41.37(c)(1)(iii).

IV. STATUS OF AMENDMENTS

Claims 1-13 and 18-20 were finally rejected in the June 16, 2006 final Office Action. In their August 15, 2006 Amendment After Final Action, Appellant sought to amend claims 1-4, 7, 11-13, and 20. The Examiner stated in the September 1, 2006 Advisory Action that, for purposes of appeal, the amendments included in Appellant's August 15, 2006 Amendment would be entered. Submitted concurrently with this Appeal Brief is an amendment under 37 C.F.R. § 1.116 that amends claim 18 and cancels claim 20. Therefore, claims 1-13, 18, and 19 should be considered in this Appeal. 37 C.F.R. § 41.37(c)(1)(iv).

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claimed invention on appeal is directed to certain transgenic mice, certain isolated nucleic acid constructs, and certain methods for obtaining certain transgenic mice.

Independent claim 1 is directed to transgenic mice whose genome comprise a nucleic acid construct that comprises a reporter nucleic acid encoding a reporter operably linked to a promoter comprising an androgen response element (ARE) and an androgen receptor nucleic acid encoding an androgen receptor, wherein expression of the reporter nucleic acid is regulated by expression of the androgen receptor nucleic acid, and wherein the androgen receptor nucleic acid is expressed in the mouse in at least one tissue selected from lung, heart, liver, testis, bone, prostate, and kidney such that the mouse has enhanced expression of androgen receptor relative to a wild type mouse in the at least one tissue. See, *inter alia,* page 5, lines 22-28; page 11, lines 19-25; and page 14, lines 15-21. Claims 2-7, 12, 13, and 18 ultimately depend from claim 1.

Independent claim 8 is directed to isolated nucleic acid constructs that comprise a reporter nucleic acid encoding a reporter operably linked to a promoter comprising an androgen response element (ARE), the constructs further comprising an androgen receptor nucleic acid encoding an androgen receptor, wherein expression of the reporter nucleic acid is regulated by expression of the androgen receptor nucleic acid. See, *inter alia,* page 6, lines 3-9. Claims 9 and 10 depend from claim 8.

Independent claim 11 is directed to methods for obtaining a transgenic mouse whose genome comprises a nucleic acid construct that comprises a reporter nucleic acid encoding a reporter operably linked to a promoter comprising an androgen response element (ARE) and an androgen receptor nucleic acid encoding an androgen receptor, wherein expression of the

reporter nucleic acid is regulated by expression of the androgen receptor nucleic acid, and wherein the androgen receptor nucleic acid is expressed in the mouse in at least one tissue selected from lung, heart, liver, testis, bone, prostate, and kidney, such that the mouse has enhanced expression of androgen receptor relative to a wild type mouse in the at least one tissue, wherein the mouse can be bred to produce progeny mice whose genomes comprise the nucleic acid construct, the method comprising the steps of: (a) isolating a fertilized egg from a first female mouse; (b) transferring a transgene comprising the nucleic acid construct into the fertilized egg; (c) transferring the fertilized egg of step (b) to the uterus of a pseudopregnant second female mouse; and (d) maintaining the second female mouse such that: (i) the second female mouse becomes pregnant with an embryo derived from the fertilized egg of step (c); (ii) the embryo develops into the transgenic mouse; and (iii) the transgenic mouse is viably born from the second female mouse; wherein the genome of the transgenic mouse comprises the nucleic acid construct and wherein the mouse can be bred to produce progeny mice whose genomes comprise the nucleic acid construct. See, *inter alia,* page 6, lines 10-30; page 11, lines 19-25; and page 14, lines 15-21. Claim 19 depends from claim 11.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following grounds of rejection are to be reviewed on this appeal:

1. Whether claims 1-13, 18, and 19 meet the utility requirement of 35 U.S.C. § 101.

2. Whether one of ordinary skill in the art would know how to use the claimed invention of claims 1-13, 18, and 19 so as to satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph.

3. Whether claim 18 is indefinite for lacking antecedent basis under 35 U.S.C. § 112, second paragraph. 37 C.F.R. § 41.37(c)(1)(vi).

VII. ARGUMENT

A. The Utility Rejection

1. The Legal Standard for Utility

To meet the utility requirement of 35 U.S.C. §§ 101 and 112, first paragraph, a patent applicant need only show that the claimed invention has "practical utility," *Anderson v. Natta*, 480 F.2d 1392, 1396-97, 178 USPQ 458, 461 (C.C.P.A. 1973) and provides a "specific benefit" to the public. *Brenner v. Manson*, 383 U.S. 519, 534-35, 148 USPQ 689, 695 (1966). As discussed by the Federal Circuit in *Juicy Whip Inc. v. Orange Bang Inc.*, 185 F.3d 1364, 1366, 51 USPQ2d 1700, 1702 (Fed. Cir. 1999), this is not a high threshold:

> An invention is "useful" under section 101 if it is capable of providing some identifiable benefit. See *Brenner v. Manson*, 383 U.S. 519, 534 [148 USPQ 689] (1966); *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1571 [24 USPQ2d 1401] (Fed. Cir. 1992) ("to violate Section 101 the claimed device must be totally incapable of achieving a useful result"); *Fuller v. Berger*, 120 F. 274, 275 (7th Cir. 1903) (test for utility is whether invention "is incapable of serving any beneficial end").

Although an asserted utility must be described with specificity, a patent applicant need not demonstrate utility to a certainty. In *Carl Zeiss Stiftung v. Renishaw PLC*, 945 F.2d 1173, 1180, 20 USPQ2d 1094, 1100 (Fed. Cir. 1991), the Federal Circuit explained:

> An invention need not be the best or only way to accomplish a certain result, and it need only be useful to some extent and in certain applications: "[T]he fact that an invention has only limited utility and is only operable in certain applications is not grounds for finding lack of utility." *Envirotech Corp. v. Al George, Inc.*, 730 F.2d 753, 762, 221 USPQ 473, 480 (Fed. Cir. 1984).

An asserted utility is specific if it shows that the claimed invention can be used to provide a well defined and particular benefit to the public. *In re Fisher*, 421 F.3d 1365, 1371, 76 USPQ2d 1225, 12261 (Fed. Cir. 2005). The Utility Guidelines set forth in the MPEP also define a "specific" utility as one that is particular to the subject matter claimed and would not be applicable to a broad class of inventions. MPEP § 2107.01. The "specific" utility requirement is met unless the asserted utility is "so vague as to be meaningless" and amounts to a "nebulous expression" such as "biological activity" or "biological properties" that does not convey meaningful information about the utility of what is being claimed. *See In re Fisher*, 421 F.3d at 1371; *see also Cross v. Iizuka*, 753 F.2d 1040, 1048, 224 USPQ 739, 745 (Fed. Cir. 1985).

If a claimed invention meets at least one stated objective, utility under § 101 is clearly shown. *Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 958, 220 USPQ 592, 598 (Fed. Cir. 1983). Proof of one of the disclosed utilities suffices to meet the statutory utility requirement. *See Standard Oil Co. v. Montedison, S.p.a.*, 664 F.2d 356, 375, 212 USPQ 327, 343 (3d Cir. 1981); s*ee also Krantz v. Olin*, 356 F.2d 1016, 1019, 148 USPQ 659, 661-62 (C.C.P.A. 1966); *E.I. du Pont de Nemours & Co. v. Berkeley & Co.*, 620 F.2d 1247, 1258 n.10, 205 USPQ 1, 8 n.10 (8th Cir. 1980). Any utility of a claimed compound is sufficient to satisfy 35 U.S.C. § 101. *Cross*, 753 F.2d at 1051, 224 USPQ at 748.

The MPEP sets forth similar guidelines for Examiners. It states that the patent laws only require that applicants establish one specific, substantial and credible utility in order to satisfy the utility requirement, and makes no restrictions on the number of utilities that may be asserted by an applicant:

> It is common and sensible for an applicant to identify several specific utilities for an invention, particularly where the invention is a product (e.g., a machine, an article of manufacture or a composition of matter). However, regardless of the category of invention that is claimed (e.g., product or process), an applicant need only make ***one credible assertion of specific utility*** for the claimed invention to satisfy 35 U.S.C. 101 and 35 U.S.C. 112; ***additional statements of utility, even if not "credible," do not render the claimed invention lacking in utility***. See, e.g., *Raytheon v. Roper*, 724 F.2d 951, 958, 220 USPQ 592, 598 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 (1984) ("When a properly claimed invention meets ***at least one stated objective***, utility under 35 U.S.C. 101 is clearly shown."); *In re Gottlieb*, 328 F.2d 1016, 1019, 140 USPQ 665, 668 (C.C.P.A. 1964) ("Having found that the antibiotic is ***useful for some purpose***, it becomes ***unnecessary to decide whether it is in fact useful for the other purposes 'indicated' in the specification as possibly useful.***"); *In re Malachowski*, 530 F.2d 1402, 189 USPQ 432 (C.C.P.A. 1976); *Hoffman v. Klaus*, 9 USPQ2d 1657 (Bd. Pat. App. & Inter. 1988). Thus, if applicant makes ***one credible assertion*** of utility, utility for the claimed invention as a whole is established.

See MPEP § 2107.02 (emphasis added).

In addition to conferring a specific benefit on the public, the benefit must also be "substantial." *Brenner*, 383 U.S. at 534, 148 USPQ at 695. A "substantial" utility is a practical, "real-world" utility. *Nelson v. Bowler*, 626 F.2d 853, 856, 206 USPQ 881, 883 (C.C.P.A. 1980). Courts have used the labels "practical utility" and "real world" utility interchangeably in determining whether an invention offers a "substantial utility." *In re Fisher*, 421 F.3d at 1371. The C.C.P.A. has stated:

> Practical utility is a shorthand way of attributing "real world" value to claimed subject matter. In other words, one skilled in the art can use a claimed discovery in a manner which provides some immediate benefit to the public.

Nelson, 626 F. 2d at 856, 206 USPQ at 883. Therefore, to satisfy the "substantial" utility requirement, an applicant must show that the claimed invention has a significant and presently available benefit to the public. *In re Fisher*, 421 F.3d at 1371.

2. The Specification Provides A Credible, Specific and Substantial Utility For The Invention of Claims 1-13, 18, and 19

The Examiner has finally rejected claims 1-13, 18, and 19 under 35 U.S.C. § 101 for allegedly lacking utility. The Examiner does not dispute that androgen receptor is an important target in multiple areas of drug discovery and patient therapy, as recited in the specification, *inter alia,* at page 4, lines 4-15. Rather, the Examiner contends that the claimed transgenic mice do not have a phenotype that distinguishes them from wild type mice and, on this basis, concludes that the claimed invention is not supported by a specific utility. Appellant disagrees.

Appellant respectfully submits that the specification, as filed, does indeed establish a credible, specific, and substantial utility for the claimed invention. Independent claims 1 and 11 require that the androgen receptor nucleic acid is expressed in the transgenic mouse in at least one tissue selected from the group consisting of lung, heart, liver, testis, bone, prostate, and kidney, such that the mouse has enhanced expression of androgen receptor relative to a wild type mouse in the at least one tissue. Accordingly, the transgenic mouse of the invention has enhanced expression of androgen receptor relative to wild type mouse in the at least one tissue. The constructs of independent claim 8 can be used to produce the transgenic mice of claims 1 and 11.

As stated in the specification, the transgenic mice of the invention provide a model system to monitor activity of androgen receptor in different organs and tissues. The cells

in which the nucleic acid reporter is expressed can be readily determined by bioluminescence imaging techniques. These transgenic mice are useful for the development of pharmaceutical agents for the treatment of disorders associated with aberrant, up-regulated androgen expression relative to a wild type mouse. In particular, agents can be screened to find those that inhibit or activate the activity of the androgen receptor as measured by the reporter.

The claimed invention has the credible, specific, and substantial use of studying the tissue selective activity of pharmacological agents by inhibition or activation of androgen receptor that is overexpressed in certain tissues. This is not a study of the properties of the transgenic mouse itself but, rather, a study of the ability of an agent to inhibit or activate androgen receptor expression in certain tissues. This use is both credible and specific given that, as is well known in the art and stated in the specification, the androgen receptor is a hormone regulated transcription factor that controls the expression of many genetic programs involved in normal physiological processes as well as in pathological conditions such as cancer. In addition, this is a substantial utility in that it is a real world use (i.e., the identification of androgen receptor modulating agents for the treatment of androgen receptor mediated disorders) rather than studying the properties of the claimed transgenic mouse product itself.

For all of the above reasons, Appellant respectfully submits that the final rejection of claims 1-13, 18, and 19 under 35 U.S.C. § 101 should be reversed.

B. The Enablement Rejection

1. The Legal Standard For Enablement

To satisfy the enablement requirement under 35 U.S.C.§ 112, first paragraph, the specification must describe how to make and use the claimed invention without undue

experimentation. *See Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916); *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988); *see also* MPEP § 2164. However, a patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986); and *Lindemann Maschinenfabrik GMBH v. Am. Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. § 112 is satisfied. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (C.C.P.A. 1970). However, failure to disclose other methods by which the claimed invention may be made does not render a claim invalid under 35 U.S.C. § 112. *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524, 1533, 3 USPQ2d 1737, 1743 (Fed. Cir. 1987).

If a statement of utility in the specification contains within it a connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated, 35 U.S.C. § 112 is satisfied. *In re Johnson*, 282 F.2d 370, 373, 127 USPQ 216, 219 (C.C.P.A. 1960); *In re Hitchings*, 342 F.2d 80, 87, 144 USPQ 637, 643 (C.C.P.A. 1965). *See also In re Brana,* 51 F.3d at 1566, 34 USPQ2d at 1441.

The Federal Circuit and its predecessor court has made plain that the utility requirement of 35 U.S.C. § 101, and the "how to use" requirement of 35 U.S.C. § 112, first paragraph, have the same basis -- specifically, the disclosure of a credible utility. *In re Brana*,

51 F.3d at 1564 n.12, 34 USPQ2d at 1439 n.12; *In re Jolles*, 628 F.2d at 1326 n.10, 206 USPQ at 889 n.10; *In re Fouche*, 439 F.2d at 1243,169 USPQ at 434.

2. The Skilled Worker Would Know How To Use The Invention Of Claims 1-13, 18, and 19

The Examiner has finally rejected claims 1-13, 18, and 19 under 35 U.S.C. § 112, first paragraph stating that since the claimed invention is not supported by either a clear asserted utility or a well-established utility, one skilled in the art would not know how to use the invention. Appellant disagrees.

In support of the enablement rejection, the Examiner relied upon three references (Appendix B) in the December 29, 2005 Office Action as purportedly teaching that making transgenic animals is unpredictable.

Hammer et al., 1990, Cell, 6: 1099-1112 ("Hammer et al., 1990") is cited as purportedly providing a teaching that there is unpredictability in using transgene constructs amongst different animals. Appellant respectfully submits that Hammer et al. 1990 (a 1990 publication) does not represent the state of the art of Appellant's invention. In addition, Appellant respectfully points out that claims 1-7, 11-13, 18, and 19 are directed to transgenic mice, an isolated cell or cells from transgenic mice, methods for obtaining or producing transgenic mice, or methods of screening using transgenic mice, and thus are directed to mice and not different, non-mouse animals with which Hammer et al. 1990 is applied. Claims 8-10 are directed to isolated nucleic acid constructs useful for producing, inter alia, the claimed transgenic mice and thus it should be apparent that the subject matter of claims 8-10 is also enabled.

Cowan et al., 2003, Xenotransplantation, 10: 223-231 ("Cowan et al.") is cited as purportedly providing a teaching that there is unpredictability in using promoters in different animals, such as mice and pigs. For the reasons discussed in the preceding paragraph, Appellant submits that the claims are enabled in view of Cowan et al.

Hammer et al., 1986, J. of Anim. Sci., 63: 269-278 ("Hammer et al., 1986") is cited as purportedly providing a teaching that there is unpredictability in the behavior of a gene of interest expressed in different animals, such as mice and pigs. For the reasons discussed above with regard to Hammer et al., 1990, Appellant submits that the claims are enabled.

Appellant respectfully submits that the specification of the instant application discloses to the skilled worker how to make and use the claimed invention. The arguments detailed, *supra,* in Section **A2** concerning the utility of the claimed invention are relevant here. The Federal Circuit and its predecessor court has made plain, the utility requirement of 35 U.S.C. § 101, and the "how to use" requirement of 35 U.S.C. § 112, first paragraph, have the same basis -- specifically, the disclosure of a credible utility. *In re Brana*, 51 F.3d at 1564 n.12, 34 USPQ2d at 1439 n.12; *In re Jolles*, 628 F.2d at 1326 n.10, 206 USPQ at 889 n.10; *In re Fouche*, 439 F.2d at 1243,169 USPQ at 434. And, as discussed above, several credible utilities are disclosed in the application as filed.

Appellant has shown that claims 1-13, 18, and 19 have a specific, substantial and credible utility, as detailed in section **A2**, *supra*. Therefore, the rejection of claims 1-13, 18, and 19 under 35 U.S.C. § 112, first paragraph, cannot stand.

C. The Indefiniteness Rejection

Claim 18 was rejected under 35 U.S.C. § 112, second paragraph, as lacking antecedent basis. Appellant submits that the amendment to claim 18 submitted concurrently with this Appeal Brief provides antecedent basis for claim 18 dependency to claim 1. Therefore, the rejection of claim 18 under 35 U.S.C. § 112, second paragraph cannot stand.

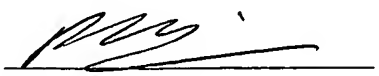
VIII. CLAIMS APPENDIX

Appendix A sets forth claims 1-13, 18, and 19 which are pending in this application and are on appeal. 37 C.F.R. § 41.37(1)(c)(viii).

IX. CONCLUSION

For all of the reasons set forth herein, Appellant respectfully submits that the various rejections of claims 1-13, 18, and 19 are erroneous and requests that the Board overturn them. All of the pending claims should be allowed.

Respectfully submitted,



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Date: March 20, 2007

CLAIMS APPENDIX A

CLAIMS 1-13, 18, AND 19, ON APPEAL

Claim 1: A transgenic mouse whose genome comprises a nucleic acid construct, wherein said construct comprises a reporter nucleic acid encoding a reporter operably linked to a promoter comprising an androgen response element (ARE), and said construct further comprises an androgen receptor nucleic acid encoding an androgen receptor, wherein expression of said reporter nucleic acid is regulated by expression of said androgen receptor nucleic acid, and wherein said androgen receptor nucleic acid is expressed in said mouse in at least one tissue selected from the group consisting of lung, heart, liver, testis, bone, prostate, and kidney, such that said mouse has enhanced expression of androgen receptor relative to a wild type mouse in said at least one tissue.

Claim 2: The transgenic mouse of claim 1 wherein said reporter is luciferase.

Claim 3: The transgenic mouse of claim 1 wherein said androgen response element is 2XDR-1.

Claim 4: A cell isolated from the transgenic mouse of claim 1, wherein the genome of said cell comprises said nucleic acid construct.

Claim 5: The cell of claim 4 wherein said reporter is luciferase.

Claim 6: The cell of claim 4 wherein said androgen response element is 2XDR-1.

Claim 7: A cell line comprising the cell of claim 4.

Claim 8: An isolated nucleic acid construct that comprises a reporter nucleic acid encoding a reporter operably linked to a promoter comprising an androgen response element (ARE), and said construct further comprises an androgen receptor nucleic acid encoding an androgen receptor, and wherein expression of said reporter nucleic acid is regulated by expression of said androgen receptor nucleic acid.

Claim 9: The construct of claim 8 wherein said reporter is luciferase.

Claim 10: The construct of claim 8 wherein said androgen response element is 2XDR-1.

Claim 11: A method for obtaining a transgenic mouse whose genome comprises a nucleic acid construct, wherein said construct comprises a reporter nucleic acid encoding a reporter operably linked to a promoter comprising an androgen response element (ARE), and

said construct further comprises an androgen receptor nucleic acid encoding an androgen receptor, wherein expression of said reporter nucleic acid is regulated by expression of said androgen receptor nucleic acid, and wherein said androgen receptor nucleic acid is expressed in said mouse in at least one tissue selected from the group consisting of lung, heart, liver, testis, bone, prostate, and kidney, such that said mouse has enhanced expression of androgen receptor relative to a wild type mouse in said at least one tissue,

wherein said mouse can be bred to produce progeny mice whose genomes comprise said nucleic acid construct, said method comprising the steps of:

(a) isolating a fertilized egg from a first female mouse;

(b) transferring a transgene comprising said nucleic acid construct into the fertilized egg;

(c) transferring the fertilized egg of step (b) to the uterus of a pseudopregnant second female mouse; and

(d) maintaining said second female mouse such that:

(i) said second female mouse becomes pregnant with an embryo derived from said fertilized egg of step (c);

(ii) said embryo develops into said transgenic mouse; and

(iii) said transgenic mouse is viably born from said second female mouse;

wherein the genome of said transgenic mouse comprises said nucleic acid construct and wherein said mouse can be bred to produce progeny mice whose genomes comprise said nucleic acid construct.

Claim 12: A method for producing a transgenic mouse cell line that expresses a reporter nucleic acid, said method comprising:

(a) isolating cells from the transgenic mouse of claim 1; and

(b) placing the isolated cells under conditions to maintain growth and viability of the isolated cells such that said transgenic mouse cell line expresses said reporter nucleic acid.

Claim 13: A method of screening for a modulator of the androgen receptor, comprising administering a test substance to the transgenic mouse of claim 1 and assaying the effect of said test substance on the activity of the androgen receptor.

Claim 18: The transgenic mouse of claim 1 wherein said nucleic acid construct comprises SEQ ID NO:1.

Claim 19: The method of claim 11 wherein said nucleic acid construct comprises SEQ ID NO:1.

EVIDENCE APPENDIX B

Appellant states that the following evidence relied upon by the Examiner for rejection of the instant claims was presented in the Office Action mailed December 29, 2005, pages 9-10:

Hammer et al., 1990, Cell, 6: 1099-1112

Cowan et al., 2003, Xenotransplantation, 10: 223-231

Hammer et al., 1986, J. of Anim. Sci., 63: 269-278

RELATED PROCEEDINGS APPENDIX C

NONE

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF
RICARDO ATTAR, ET AL
APPLICATION NO: **10/620,514**
FILED: **07/16/2003**
FOR: **TRANSGENIC NON-HUMAN MAMMALS EXPRESSING A REPORTER NUCLEIC ACID UNDER THE REGULATION OF ANDROGEN RESPONSE ELEMENTS**

ART UNIT: **1632**
EXAMINER: **HAMA, JOANNE**
CONFIRMATION NO: **3956**

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

TRANSMITTAL LETTER

Sir:

Enclosed herewith is one copy of an Amended Appeal Brief in the above-identified application.

☒ Please charge Deposit Account No. 19-3880 in the name of Bristol-Myers Squibb Company in the amount of $500 for payment of the appeal fee. An additional copy of this paper is here enclosed. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Account No. 19-3880 in the name of Bristol-Myers Squibb Company.

☐ Enclosed is a Petition for Extension of Time.

Respectfully submitted,

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Date: March 20, 2007

GENETIC ENGINEERING OF MAMMALIAN EMBRYOS[1,2,3]

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ABSTRACT

A gene consisting of the mouse metallothionein I promoter/regulator (MT) fused to the human growth hormone (hGH) structural gene (MThGH) was microinjected into rabbit, sheep and pig eggs. Visualization of nuclear structures was accomplished by interference-contrast (I-C) microscopy for rabbit and sheep eggs and by centrifugation and I-C microscopy for pig eggs. The gene integrated into the chromosomes of each species with an efficiency of 13% in rabbits, 1% in sheep and 10% in pigs. Human GH mRNA was detected in the liver of transgenic rabbits as well as tail and ear samples of pigs. Immunoreactive hGH was present in the serum of a transgenic rabbit and plasma of most transgenic pigs. In several pigs hGH levels increased between birth and 90 d of age. The presence of substantial quantities of hGH in plasma of pigs did not increase postnatal somatic growth rates. Founder animals will be bred and their transgenic and control progeny used to assess the effects of hGH on feed efficiency and carcass composition. These experiments demonstrate the feasibility of introducing foreign genes into the genome of several animal species by microinjection of eggs.

(Key Words: Rabbits, Sheep, Pigs, Somatotropin, Genetic Engineering, Genes.)

Introduction

A variety of genes, particularly those involved in regulation of growth, have been introduced into mice. The rat and human growth hormone (GH) structural genes become stably integrated into the mouse genome, but they are not efficiently expressed (Hammer et al., 1984a). In contrast, transgenic mice harboring fusion genes consisting of the mouse metallothionein promoter/regulator (MT) fused to the rat (r), bovine (b) or human (h) GH structural gene exhibit high, metal inducible levels of GH mRNA in many organs, particularly the liver and intestine (Palmiter et al., 1982, 1983; Hammer et al., 1985a). Most of the mice have substantial levels of foreign GH in their sera and show markedly enhanced growth rates and a doubling in adult body size. The genes generally become stably integrated into the germline and are heritable with the transgenic offspring exhibiting enhanced growth.

The application of gene transfer techniques to domestic species has been impeded primarily by the inability to visualize egg nuclei. By using interference-contrast microscopy (I-C) for rabbit and sheep eggs (Hammer et al., 1985c), and centrifugation and I-C microscopy for pig and cow eggs (Wall et al., 1985), nuclei become visible and can be microinjected. The purpose of this study was to determine if microinjected genes could become incorporated into the genome of other mammalian species and to determine if hGH gene expression would enhance growth rates. To test such possibilities, we microinjected mouse metallothionein-human growth hormone (MThGH) fusion gene into the nuclei of rabbit, sheep and pig eggs and examined the resulting animals for gene integration and expression.

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[2]We gratefully acknowledge Mary Chandlee, Dennis McDuffie, Anne Powell, Leah Schulman, Myrna Trumbauer, and Mary Yagle for their expert technical assistance and Kenneth Bender, Paul Graninger, Paul Fallon, James Piatt and David Sherman for exceptional animal care.

[3]Presented by the senior author at a symposium on "Future Impact of Biotechnology in Animal Science," held August 14, 1985 at the 77th Annu. Meet. of the Amer. Soc. of Anim. Sci., Univ. of Georgia, Athens.

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TABLE 1. SUPEROVULATION IN SHEEP AND PIGS

Group	Sheep	Pig
No. of females injected	205	218
No. of females that ovulated	196	182
No. of eggs collected/female that ovulated	10.4 ± .5[a]	23.4 ± 2.6
Egg recovery after expected time of ovulation, h[b]	18-25	18-27

[a]Mean ± SE.

[b]Ewes were expected to ovulate 50 to 55 h following sponge removal; gilts were expected to ovulate 40 to 44 h after administration of human chorionic gonadotropin (Hunter and Dziuk, 1968; Hunter, 1972).

Materials and Methods

Animal Treatment. The methods of hormone administration, estrous synchronization, breeding, embryo collection and transfer for mice, rabbits, sheep and pigs have been previously reported (Hammer et al., 1985c).

Superovulation Efficiency. Progestogen-impregnated intravaginal sponges were used in combination with multiple injections of porcine follicle stimulating hormone (FSH) to obtain superovulation in ewes (Armstrong and Evans, 1983, Armstrong et al., 1983). With this regimen we obtained both a high mean ovulation rate (16.9%) and a high mean embryo yield (table 1). The relatively low embryo recovery rate (64%) was probably caused by fimbrial adhesions and scarring of oviductal tissue due to multiple use of some donors and failure to recover any eggs in some ewes that ovulated while the progesterone pessary was in place.

In pigs, standardized superovulation techniques were utilized (Wall et al., 1985). Egg yield per ovulating female was high (table 1), but 11.5% of female failed to ovulate and 11% developed cystic ovaries, some of which ovulated. The number of ovulation points per gilt could not be determined accurately because of ovarian adhesions and scarring caused by multiple uses of some donors.

Microinjection and Nucleic Acid Analysis. A few hundred copies of a 2.6-kilobase BstEII/EcoRI linear DNA fragment isolated from a plasmid containing the MThGH gene was microinjected as described (Hammer et al., 1985c). The number of copies of the MThGH gene that integrated was estimated by quantitative dot hybridization. The DNA from some of the transgenic animals also was analyzed by Southern blotting. The MThGH mRNA was measured by solution hybridization utilizing a complementary end-labeled 21-base oligonucleotide probe (Ornitz et al., 1985).

Human Growth Hormone Analysis. Plasma samples were analyzed in duplicate at 2.5 and 10 μl by radioimmunoassay using a hGH antibody (NIAMDD-anti-hGH-1) and reference standard (NIAMDD-hGH-RP-1) provided by Dr. Raiti[7] (National Hormone and Pituitary Program) and radioiodinated (Miller et al., 1985) hGH (LER-hGH-1) provided by Dr. L. Reichert[8]. The hGH assay did not cross-react with porcine GH. Cross-reactivity with rabbit GH was not determined but plasma from non-transgenic rabbits did not cause displacement in the assay. Plasma samples were designated as negative for hGH when an hGH concentration was less than 2 ng/ml (approximately 15% inhibition of binding from buffer controls with 10-μl samples).

Results and Discussion

Visualization of Egg Nuclei. Visualization of nuclei in eggs of domestic livestock has been the greatest impediment to the successful application of gene transfer by microinjection. Nuclei of mouse and rabbit eggs (figure 1a) are easily discernible with conventional light microscopic techniques. However, the cytoplasm of sheep eggs and, particularly, pig and cow eggs is so optically dense that other methods for visualization had to be developed. Deoxyribonucleic acid-specific fluorochromes have recently been utilized to localize nuclei in cow

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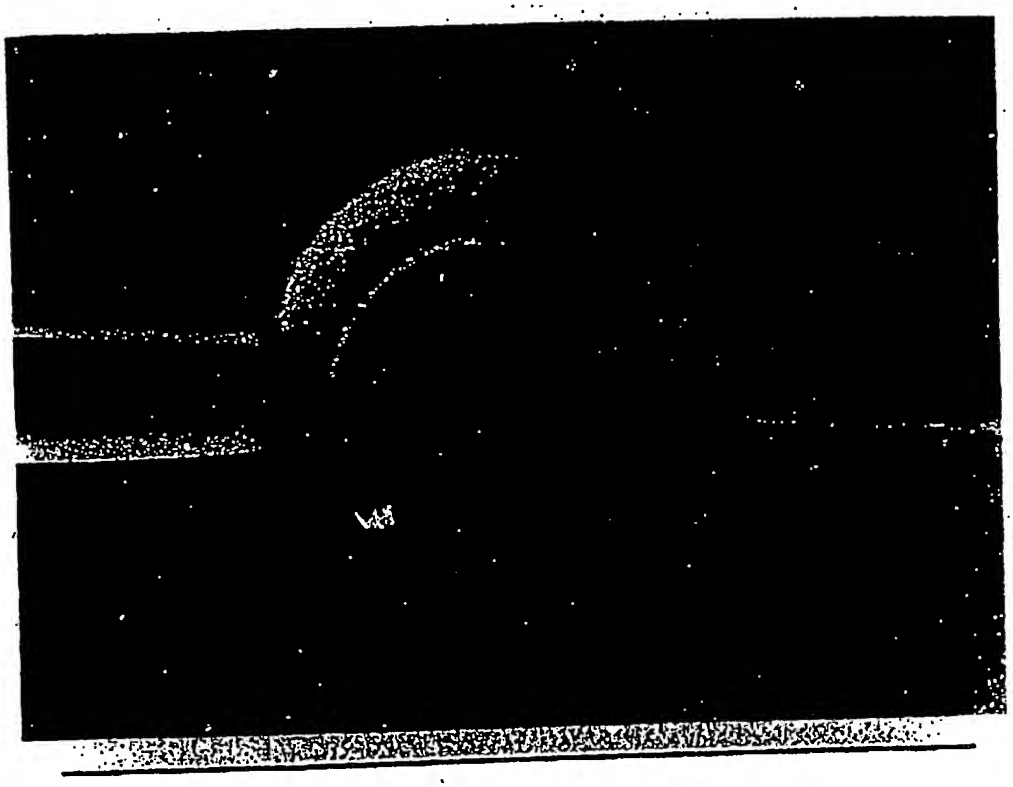

(Crister et al., 1983; Minhas et al., 1984), pig (Pursel et al., 1985) and sheep eggs (R. E. Hammer, C. E. Rexroad and R. L. Brinster, unpublished observations). However, the applications of these techniques as an aid to microinjection seems limited because short exposure of Hoechst 33342- and 33258-stained pig (Wall et al., 1985) and sheep eggs to ultraviolet light (100 W) was embryotoxic.

We found that sheep egg nuclei can be located with the aid of I-C microscopy (figure 1b). Examination of fertilized sheep eggs by I-C microscopy and fluorescent microscopy using a DNA-specific fluorochome indicated that at least 80% of fluorescent pronuclei were visible with I-C microscopy. Pig and cow ova are so optically opaque that neither phase, dark field nor I-C microscopy (figure 2a) are useful for visualizing nuclei. Centrifugation has been used to stratify the cytoplasm of sea urchin embryos into a number of distinct layers, one of which contains the nucleus and is devoid of lipid and pigment (Harvey, 1956). Therefore, we explored the use of centrifugation to aid in visualizing nuclei in pig, cow and sheep eggs (Wall et al., 1985). Centrifugation of pig (figure 2b) and cow (figure 3) ova at 15,000 x g for 5 to 10 min markedly stratifies the cytoplasm, leaving the pronuclei or nuclei accessible to microinjection. While the cytoplasm of sheep eggs stratifies when subjected to 15,000 x g, centrifugation does not greatly improve visualization.

Microinjection and Embryo Survival. To determine the effects of 12 h of culture and the toxicity of nuclear-injected DNA (2 to 5 pl of 2 ng/μl) on sheep and pig egg development, we transferred cultured control and microinjected eggs into recipients and examined the development of recovered eggs.

Only 26% of uninjected one- and two-cell sheep eggs and 52% of centrifuged uninjected pig eggs survived to blastocysts (table 2). Such a low percentage of development may be due, at least in part, to the trauma of extended culture. Injection of DNA further reduced embryo development by approximately 50% in each species (table 2).

As indicated by a decrease in the number of eggs that developed to the morula/blastocyst stage in culture, and the lower recovery of injected eggs as fetuses from pseudopregnant females, microinjection of buffer into the male pronucleus of one-cell eggs was detrimental to development. Injection of a DNA dose (2 ng/μl) that provides a high integration efficiency (27%) further diminishes mouse egg survival to blastocysts and fetuses (Brinster et al., 1985).

Integration Efficiency. In mice, both the form and concentration of DNA, as well as the site of gene injection, are important factors for efficient integration (Brinster et al., 1985). Linear molecules of DNA integrate with much higher efficiency than circular forms, and nuclear deposition of DNA is more effective than cytoplasmic deposition. Because of our initial difficulty in visualizing pig egg nuclei we piloted gene transfer in pigs by cytoplasmic injection of one- and two-cell eggs. None of the fetuses that developed from these eggs contained integrated MThGH sequences (table 3). In contrast, nuclear injection of centrifuged pig eggs resulted in an integration efficiency of approximately 10%. Integration frequency was 12.8 and 1.3% in the rabbit and sheep, respectively (table 3).

These efficiencies of integration, particularly in the sheep, are much lower than rates we currently obtain in mice (27%). Because of the difficulty and expense in obtaining large numbers of pig and sheep eggs, it has been difficult to examine factors which may influence integration rate. The frequency of integration for these species (table 3) has resulted from using conditions that were shown to be optimal for mouse eggs (Brinster et al., 1985). Both the age of the ovum and the structure of the host chromosomes may be important factors for integration. Efficiency in the rabbit has been improved to approximately 30% by injecting eggs in the early pronuclear stage (data not shown), as compared with injecting near the time of one-cell cleavage. Such considerations may be equally important in other species.

Reasons for the extremely low integration efficiency in sheep is puzzling. Although none of the lambs that developed from cytoplasmically injected eggs contained integrated DNA (data not shown), we anticipated markedly improved efficiency when we began microinjecting into the nucleus. Such a dramatic increase has not occurred.

The pattern of DNA integration in MThGH rabbits and pigs was similar to that observed in mice. The number of copies of the integrated gene varied from 1 to 490 per cell. Southern analysis verified that all the transgenic rabbits and pigs contained intact copies of MThGH,

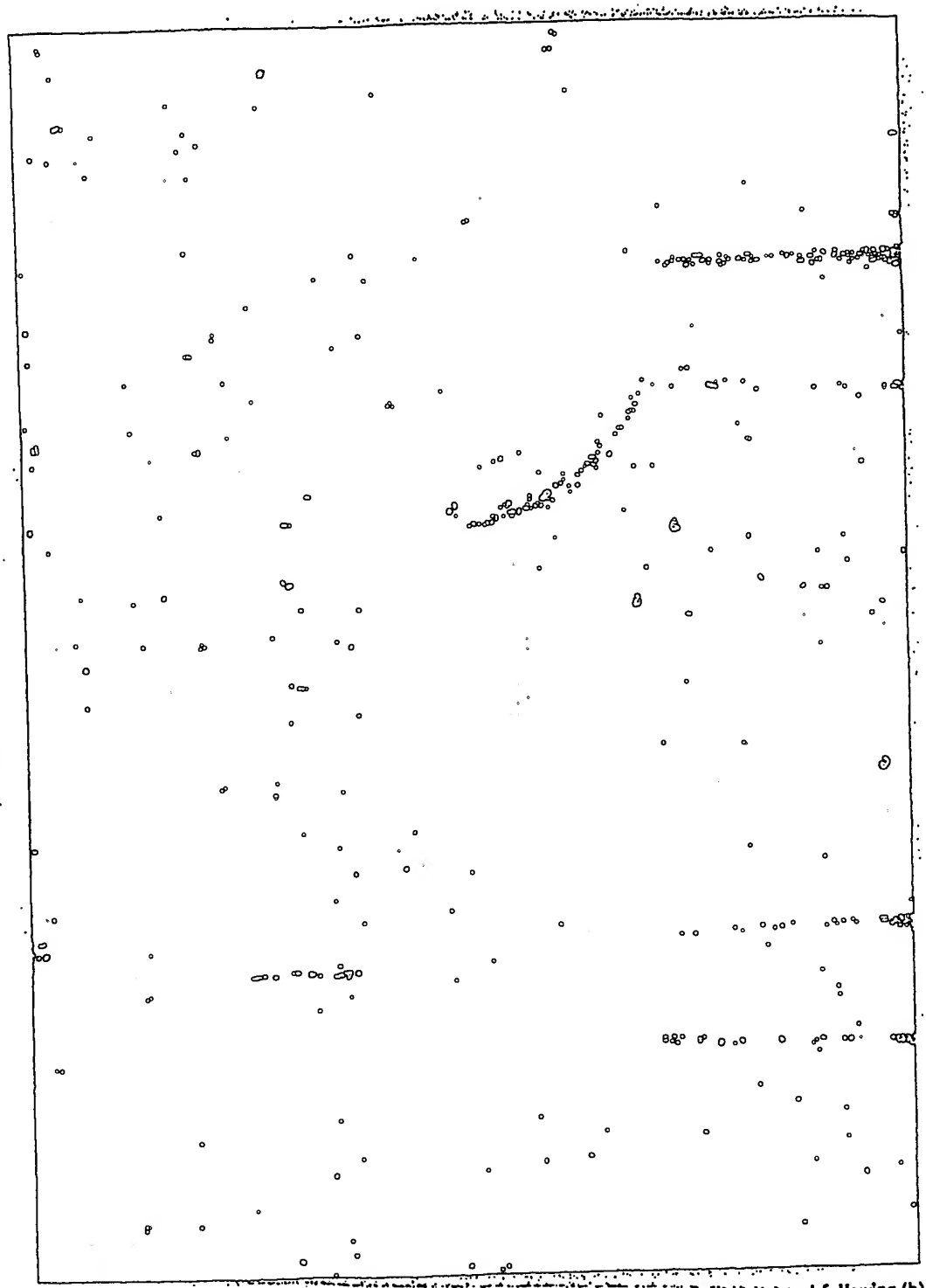

Figure 2. Interference-contrast photomicrographs of one-cell fertilized pig ova before (a) and following (b) centrifugation and microinjection. Porcine ova have been centrifuged at 15,000 × g for 3 min to reveal the pronuclei.

Figure 3. Interference-contrast photomicrograph of a two-cell cow egg following microinjection. The injecting pipette is seen within the nucleus of a blastomere following injection.

with many oriented in tandem head-to-tail arrays, as well as some in head-to-head configuration. Restriction analysis of DNA isolated from the one transgenic sheep indicated that the MThGH gene was not intact.

Expression of MThGH Genes. The mouse metallothionein gene is expressed in many tissues, particularly in large secretory organs such as liver, kidney and intestine. The gene has a transcriptional promoter that is regulated by heavy metals such as cadmium and zinc. The promoter functions when transfected into human (Mayo et al., 1982) and rabbit (R. D. Palmiter, unpublished observations) tissue culture cells, and has been shown to provide regulatable expression of the rat, bovine and human GH genes in transgenic mice. These results led us to choose this promoter to begin our work in introducing genes into other mammalian species.

Four of the 16 MThGH rabbits had detectable levels of hGH mRNA in the liver and one animal (200-3) had substantial levels (table 4). Tissue-specific expression was examined in more detail in one of the founder rabbits (163-3). The small intestine contained the

TABLE 2. EFFECT OF DNA MICROINJECTION ON EMBRYO SURVIVAL TO BLASTOCYST

Group	Sheep	Pig
Uninjected[a]	28/108 (26)[c]	22/42 (52)
Microinjected[ab] with DNA	24/233 (10)	39/165 (23)

[a]Only eggs with visible pronuclei or nuclei were used. All pig eggs were centrifuged to permit visualization of nuclei. Both uninjected and injected eggs were in culture tubes and(or) microdrops for approximately 12 h before transfer into recipient ewes or gilts. Eggs were recovered from sheep at 8 d and pigs at 6 d following transfer. Recovered eggs having a visible cavity were classified as blastocysts.

[b]Pronuclei or nuclei of sheep and pig eggs were microinjected with a solution containing 2 ng/μl of MThGH genes. Eggs were transferred approximately 3 to 5 h following microinjection of DNA.

[c]Blastocysts per eggs transferred. Numbers in parentheses represent percent.

highest quantity of hGH mRNA, with the liver, kidney and testis containing lower but detectable levels (data not shown). In pigs, the hGH mRNA content of tail or ear samples was quantitated to avoid any adverse consequences resulting from partial hepatectomy. Nevertheless, MThGH mRNA was detectable in many of the founder pigs (table 5). A more detailed examination of tissue expression in pigs will be undertaken once founder animals have been bred and germ-line transmission has been verified.

To determine if MThGH expression resulted in production of immunoassayable hGH, we quantitated hGH levels in the serum of a transgenic rabbit and the plasma of transgenic pigs. A rabbit had a substantial level of serum hGH. Eleven of the 18 founder pigs had detect-

TABLE 3. EFFICIENCY OF GENE TRANSFER INTO ANIMALS

Species	Site of injection	Eggs[a] injected and transferred	Fetuses and neonates[b]	Animals with integrated gene[c]	Animals expressing the gene[d]
Mice[e]	Cytoplasm	890	224 (25)	2 (1)	ND
	Pronucleus	946	111 (12)	39 (35)	23 (59)
Rabbit	Pronucleus	1,907	218 (11)	28 (13)	4 (25)
Sheep	Pronucleus	1,032	73 (7)	1 (1)	ND
Pig	Cytoplasm	485	42 (9)	0 (0)	
	Pronucleus	2,035	192 (9)	20 (10)[f]	11[g] (61)

[a]Eggs were microinjected with a solution of linear DNA containing MThGH genes.

[b]The number of animals (fetuses, stillborns and neonates) resulting from injected ova. Numbers in parentheses are the number of animals produced/total eggs injected.

[c]The number of animals that incorporated the injected DNA. Numbers in parentheses are the number of MThGH animals/total number of animals produced.

[d]The number of animals containing MThGH mRNA. Numbers in parentheses are the number of MThGH animals expressing the gene/total number of MThGH animals analyzed. Sixteen rabbits were analyzed for mRNA. ND = not determined.

[e]Data for mice are from Brinster et al. (1985).

[f]Integration efficiency for pigs based on combined data from female recipients bearing injected eggs only or injected and control eggs (Hammer et al., 1985c).

[g]Pigs containing detectable plasma human growth hormone. Plasma from 18 animals was analyzed for hGH.

TABLE 4. CHARACTERISTICS OF MThGH TRANSGENIC RABBITS AND SHEEP

Species	Animal[a] and sex	Gene copy, no./cell	hGH mRNA[b], molecules/cell	Immunoassayable hGH, ng/ml
Rabbit	163-3[c]	3	15	250
Rabbit	200-3	8	920	ND
Rabbit	68-3	28	39	ND
Rabbit	221-1	40	140	ND
Sheep	693-1[d]	1	ND[e]	ND

[a]Animals without identified gender were sacrificed as fetuses.

[b]Liver MThGH mRNA was quantitated.

[c]Male.

[d]Female.

[e]ND = not determined.

able levels of hGH at birth and by 90 d of age hGH had increased substantially in five of these pigs (table 5). In addition, five of the six pigs that were negative for hGH at birth had low but detectable levels at 90 d of age. In MThGH mice, hGH mRNA is present in fetal liver as early as d 13 of gestation (R. Palmiter, unpublished observation). However, the developmental pattern of MThGH expression in mice and pigs has not been fully explored.

Effect of Foreign GH on Growth. Many of the founder transgenic mice bearing MT-GH fusion constructs show up to a fourfold enhancement in growth rate during the maximum growth phase. Enhanced growth is evident by 3 wk of age and growth rates by 11 wk have begun to plateau at about twice normal body size (Palmiter et al., 1983; Hammer et al., 1984a). Although the MT promoter is responsive to heavy metals and GH mRNA levels increase as a result of metal induction, the additional GH does not further enhance growth (Hammer et al., 1984b). Thus, in mice the constitutive activity of the MT promoter provides enough foreign GH to stimulate growth maximally.

The effects of hGH on growth of transgenic rabbits has not been evaluated. The animal that had detectable hGH levels had malocculusion, which impaired food consumption. In pigs, hGH in the serum of expressing animals did not increase weight gain (table 6). Transgenic offspring and full-sib littermate controls will need to be generated to determine precisely the effect of hGH on growth rates and feed efficiency. However, these preliminary data indicate that hGH will not dramatically alter growth performance. Such findings may not be surprising since injections of recombinantly derived hGH had only a weak growth-promoting effect in pigs (Baile et al., 1983). In contrast, injections of porcine (p) GH (22 $\mu g \cdot kg^{-1}$ body $wt \cdot d^{-1}$) into pigs stimulated growth by 10% and improved feed efficiency by 4% (Chung et al., 1985). Larger doses of pGH (70 $\mu g \cdot kg^{-1}$ body $wt \cdot d^{-1}$) further enhance swine growth rates and feed efficiency (21%) and the

TABLE 5. CHARACTERISTICS OF MThGH TRANSGENIC PIGS

Animal	Sex[a]	Gene copy, no./cell	hGH mRNA, molecules/cell	Immunoassayable hGH (ng/ml)	
				Birth	90 d
11-2	M	1	0	40	20
16-9	M	1	5	65	72
21-4	M	1	2	NEG[b]	2
21-5	F	1	0	NEG	NEG
20-2	M	2	4	40	337[c]
25-4	F	2	0	NEG	2
16-3	F	3	0	NEG	32
17-4[f]	F	3	0	NEG	
18-3[d]	F	3	6	60	
23-8	F	7	41	730	2,435
16-8[e]	F	10	18	53	
25-2	F	17	0	108	584
10-4	F	23	0	NEG	3
22-1[e]	F	50	24	56	
7-3[g]	F	90	12	80	
20-8	M	110	1	2	113
3-2	M	330	26	NEG	3
3-6	M	490	53	17	48

[a] M = male, F = female.

[b] NEG = negative.

[c] Plasma sample was taken at 60 d of age.

[d] Sacrificed at birth.

[e] Stillborn.

[f] Died at 6 wk of age.

[g] Died at 3 wk of age.

TABLE 6. GROWTH PERFORMANCE OF MThGH TRANSGENIC PIGS[a]

Item	Control pigs[b]	MThGH pigs[c]	
		Non-expressors	Expressors
No. of animals	7	5	5
Avg daily gain, kg	.78 ± .03	.69 ± .02	.63 ± .04

[a]The experimental period began from an initial body weight of 30 kg and terminated at 90 kg. Values are means ± SE. Pigs were fed a corn-soybean diet containing approximately 16% protein.

[b]Control pigs were non-transgenic littermates of experimental animals.

[c]MThGH transgenic pigs are founder animals listed in table 5. Expression is based on the presence of immunoassayable plasma hGH.

linearity of such responses suggests the possibility that even higher doses may further stimulate growth performance (Rebhun et al., 1985). Thus, introducing the porcine GH gene into pigs under the control of the mouse or human MT promoter or an alternative promoter may provide sufficient plasma pGH levels to enhance growth.

Alternatively, genes coding for other peptides involved in growth regulation may be introduced into domestic species. Expression of a mouse MT-human growth hormone-releasing factor (hGRF) gene in transgenic mice increases somatic growth by elevating endogenous GH levels (Hammer et al., 1985b); in steers injections of hGRF increase serum GH concentrations (Mosley et al., 1985). Genes coding for somatomedins or insulin-like growth factors (IGF) I and II have been cloned (Jansen et al., 1983; Bell et al., 1984; Ullrich et al., 1984) and, while the consequences of IGF expression from foreign genes in mice have not yet been explored, it seems possible that enhanced growth performance will result. Such possibilities and applications in livestock await further investigation.

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Targeting gene expression to endothelium in transgenic animals: a comparison of the human ICAM-2, PECAM-1 and endoglin promoters

Cowan PJ, Shinkel TA, Fisicaro N, Godwin JW, Bernabéu C, Almendro N, Rius C, Lonie AJ, Nottle MB, Wigley PL, Paizis K, Pearse MJ and d'Apice AJF. Targeting gene expression to endothelium in transgenic animals: a comparison of the human ICAM-2, PECAM-1 and endoglin promoters. Xenotransplantation 2003; 10: 223–231. © Blackwell–Munksgaard, 2003

Abstract: It is highly likely that successful pig-to-human xenotransplantation of vascularized organs will require genetic modification of the donor pig, and in particular of donor vascular endothelium. Promoters are generally tested in transgenic mice before generating transgenic pigs. Several promoters have been used to drive endothelial cell-specific expression in mice but none have yet been tested in pigs. We compared the promoters of three human genes that are predominantly expressed in vascular endothelium: intercellular adhesion molecule 2 (ICAM-2), platelet endothelial cell adhesion molecule 1 (PECAM-1) and endoglin. Expression of human complement regulatory proteins (hCRPs), directed by each of the promoters in mice, was largely restricted to vascular endothelium and leukocyte subpopulations. However, expression from the PECAM-1 promoter was weak in liver and non-uniform in the small vessels of heart, kidney, and lung. Conversely, expression from the endoglin promoter was consistently strong in the small vessels of these organs but was absent in larger vessels. The ICAM-2 promoter, which produced strong and uniform endothelial expression in all organs examined, was therefore used to generate hCRP transgenic pigs. Leukocytes from 57 pigs containing at least one intact transgene were tested for transgene expression by flow cytometry. Forty-seven of these transgenic pigs were further analyzed by immunohistochemical staining of liver biopsies, and 18 by staining of heart and kidney sections. Only two of the pigs showed expression, which appeared to be restricted to vascular endothelium in heart and kidney but was markedly weaker than in transgenic mice produced with the same batch of DNA. Thus, in this case, promoter performance in mice and pigs was not equivalent. The weak expression driven by the human ICAM-2 promoter in pigs relative to mice suggests the need for additional regulatory elements to achieve species-specific gene expression in pigs.

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Introduction

Genetic modification of the donor pig has proved to be a useful technique in xenotransplantation. Expression of human complement regulatory proteins (hCRPs), either from their endogenous promoters [1–3] or from broadly active heterologous promoters [4–6], protects porcine cardiac and renal xenografts from hyperacute rejection by primates [1, 5, 7, 8]. Although duplicating the widespread native expression pattern of hCRPs may be desirable [1], it is recognized that the critical tissue for expression of protective molecules is the vascular endothelium, the primary site of pathogenetic

events that culminate in xenograft rejection. Endothelial-specific promoters offer two advantages over "ubiquitous" promoters for expression of particular genes: the potential for higher expression [9], and elimination of the unpredictable effects of ectopic expression. The latter point is important in cases where unrestricted transgene expression may be deleterious or even lethal. For example, blockade of the NFκB pathway using dominant-negative forms of p65 has been proposed as a means of preventing endothelial cell (EC) activation and its proinflammatory consequences [10, 11]. However, in this case, global inhibition is likely to be problematic because of the role played by NFκB in many cellular processes [12]. Indeed, deletion of the p65 gene causes embryonic lethality [13]. Targeting of inhibitors to endothelium, perhaps in conjunction with temporal control of transgene expression [14], has inherent advantages.

The expense of producing transgenic pigs dictates that candidate endothelial-specific promoters should first be proven in transgenic mice. In vitro transfection experiments provide a guide to the specificity of an expression construct but do not reliably predict performance in vivo. This may be the result of the heterogeneity of EC, the influence of surrounding tissues on gene regulation, or the absence of distant regulatory elements in the constructs used. Promoter fragments that have demonstrated endothelial specificity in vitro but have not reproduced their native expression pattern in adult transgenic mice include Tie1 [15] and von Willebrand factor [16]. Endothelial-specific promoters that have been proven in transgenic mice include human thrombomodulin [17] and mouse vascular endothelial-cadherin [18]. However, there have been no reports of the use of these or other endothelial-specific promoters in pigs.

We have previously cloned the promoter of a human gene, intercellular adhesion molecule 2 (ICAM-2, CD102), and used it to drive EC-specific gene expression in vitro and in transgenic mice [19,20]. We have also characterized the promoters of two other human genes that are predominantly expressed in endothelium, platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31) and endoglin (CD105). PECAM-1 is present on myeloid cells, platelets, and a subpopulation of T cells, but is particularly strongly expressed on vascular EC [21]. Endoglin is also highly expressed on endothelium and cultured EC but on few other cell types [22]. Upstream fragments of both genes were cloned and shown to specify promoter activity in EC in vitro [23, 24]. The ICAM-2 and endoglin promoters have been used to deliver gene expression to blood vessels in mice using a gene therapy approach [25].

In this study, the activity and specificity of the three promoters were initially compared in vitro by transfecting endothelial and non-endothelial cultured cells with promoter/reporter constructs, and then in vivo by generating transgenic mice in which expression of hCRP(s) was placed under the control of each promoter. On the basis of this comparison, with particular emphasis on the uniformity of transgene expression on vascular endothelium, the best-performing promoter was used to generate hCRP transgenic pigs. Although pigs expressing hCRPs existed at the commencement of this study [3, 4, 6], recent data indicate that the level of transgene expression in at least some of these pigs is insufficient to deal with ongoing complement activation in the acute vascular rejection that occurs when hyperacute rejection is averted [26]. We have demonstrated in ex vivo experiments using hCD59 transgenic mouse hearts that high-level endothelial expression of hCRPs afforded better protection from human complement-mediated injury than moderate, widespread expression [27]. Co-expression of hCD55 with hCD59 provided enhanced protection to xenogeneic tissue [9], and hCD46 may provide further protection by its cooperative action with hCD55 [28]. We therefore chose to coexpress hCD55, hCD59 and hCD46-GPI (a glycosylphosphatidylinositol-linked version of hCD46) in pigs. High-level expression of more than one transgene can be achieved by coinjection of separate constructs [2,9], eliminating the need for the time-consuming process of crossbreeding of single transgenic pig lines.

Materials and methods

Plasmids

The 0.33-kilobase pair (kb) human ICAM-2 [20], 1.42-kb PECAM-1 [23] and 0.74-kb endoglin [24] promoter fragments are shown in Fig. 1. For transient transfection studies, each fragment was cloned separately into the firefly luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI, USA). The ICAM-2 promoter/luciferase reporter construct has been described previously [20]. For the generation of transgenic mice, a generic microinjection vector designed for the efficient expression of complementary DNAs (cDNAs) was used. This plasmid consists of promoter, hybrid or "universal" intron, cDNA, and SV40 early polyadenylation signal [19], and contains cloning sites to facilitate the substitution of different promoter and/or cDNA elements. Six

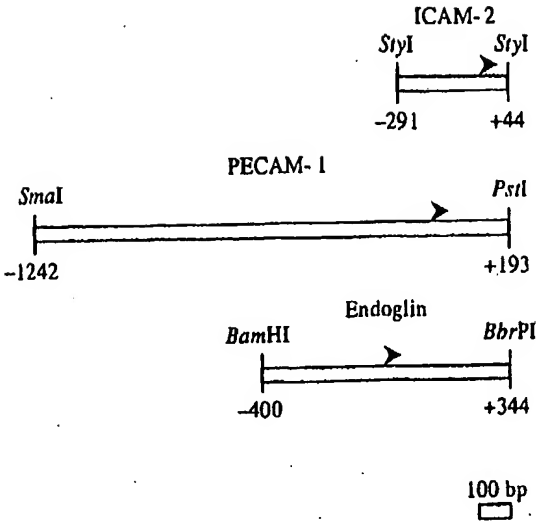

Fig. 1. Maps of the promoter fragments used in this study. Arrowheads indicate primary transcription start points in endothelial cells.

constructs containing human cDNAs were used in this study: ICAM-2 promoter/hCD55, /hCD59, and/hCD46-GPI; endoglin promoter/hCD55 and/hCD59; and PECAM-1 promoter/hCD59.. The ICAM-2 constructs have been described previously [19,20,29]. Plasmid DNA was prepared using QIAGEN plasmid kits (QIAGEN, Clifton Hill, Victoria, Australia).

Cell culture and transient transfection

Bovine aortic endothelial cells (BAEC) and COS (African green monkey kidney fibroblast) cells were cultured as previously described [20]. The SV40-immortalized porcine aortic endothelial cell line SVAP was provided by Dr I. Anegon (Nantes, France) and was grown in BAEC medium containing 10 μg/ml *cis*-hydroxyproline. Transient transfections were performed essentially as described [20] with the following changes. For SVAP and BAEC, 8 μl of LipofectAMINE, 6 μl of LipofectAMINE PLUS (Life Technologies, Gaithersburg, MD, USA) and 1.5 μg of DNA were used per well and cells were harvested after 48 h. To correct for transfection efficiency, 0.1 μg of the *Renilla* luciferase coreporter vector pRL-TK (Promega) was used. Lysates of transfected cells were assayed for firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter Assay System (Promega). Promoter activity was measured as the ratio of firefly to *Renilla* luciferase and was expressed relative to that measured for cells transfected with the promoter-less pGL3-Basic vector.

Transgenic mice and pigs

Transgenic mice and pigs were generated as described [9,30] by pronuclear microinjection of DNA at a total concentration of 10 μg/ml. Equimolar mixtures of constructs were used to produce multitransgenic animals. ICAM-2 promoter/hCD59 transgenic mice (line 237-7) have been described previously [19,20]. Transgenic animals were identified by Southern blot hybridization of tail-tip genomic DNA, prepared using the QIAamp system (QIAGEN), with the appropriate ^{32}P-labeled human cDNA probe(s).

Analysis of transgene expression

The expression of hCD46-GPI, hCD55 and hCD59 was detected using the fluorescein isothiocyanate (FITC) labeled monoclonal antibodies FITC-E4.3, FITC-IH4 and FITC-MEM43 [9, 29], respectively. Expression on peripheral blood leukocytes was analyzed by flow cytometry on a Becton Dickinson FACSCalibur as described [9, 29]. Lymphocytes, granulocytes and monocytes were distinguished on the basis of forward scatter/side scatter characteristics. Expression in tissues was examined by immunohistochemical staining of 4 μm fresh-frozen sections as described [9, 29].

Results

Promoter comparison in vitro

Promoter activity in vitro was compared by transiently transfecting porcine EC (SVAP), BAEC and simian non-endothelial (COS) cells (Fig. 2). The endoglin and ICAM-2 promoters showed strong activity in SVAP and BAEC and relatively weak activity in COS. In contrast, the PECAM-1 promoter demonstrated less specificity, showing moderate activity in SVAP, BAEC and COS. The ICAM-2 promoter was the most active of the three in porcine EC.

Promoter comparison in transgenic mice

We have previously reported that microinjection of an ICAM-2 promoter/hCD59 construct produced four transgenic mice from 27 born (15%), three of which expressed the transgene [19]. Microinjection of the PECAM-1 promoter/hCD59 construct yielded nine transgenic mice from 111 born (8%), and coinjection of the endoglin promoter/hCD55 and/hCD59 constructs produced six double-transgenic mice from 47 born (13%). Transgenics were screened for expression of hCD59 by flow

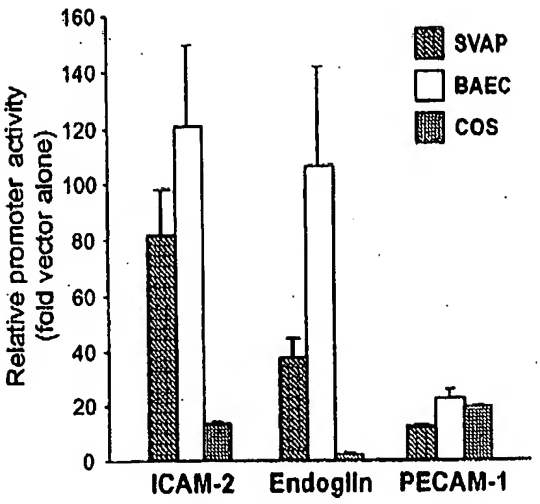

Fig. 2. Relative in vitro activity of the ICAM-2, endoglin and PECAM-1 promoters in cultured porcine endothelial cells (SVAP), bovine endothelial cells (BAEC), and simian non-endothelial cells (COS). Promoter activity was measured relative to that of vector-transfected control cells and is presented as the mean ± standard deviation of at least three independent transfections performed in duplicate.

cytometric analysis of peripheral blood leukocytes, as PECAM-1 is expressed on granulocytes, monocytes and a subpopulation of T cells [31] and endoglin is expressed on monocytes [32]. Human blood was used as a positive control, and lymphocytes, granulocytes and monocytes showed a mean fluorescence intensity (MFI) of 30, 60 and 16, respectively (data not shown). Five of the nine PECAM-1 promoter/hCD59 transgenic mice expressed hCD59 at two distinct levels on most granulocytes and monocytes, with most lymphocytes negative. The remaining mice did not express hCD59. The two levels of expression are evident in the profile of the strongest expressor, designated P59, in which positive peaks with MFI of 37 and 245 were observed (thick line, Fig. 3). Normal Mendelian inheritance of this phenotype was observed in a line established from P59, indicating that the expression pattern was not due to mosaicism. Three of the six endoglin promoter/hCD55/hCD59 double-transgenic mice expressed both hCD55 and hCD59 on a small subpopulation (approximately 15%) of lymphocytes (thin line, Fig. 3; MFI = 100 for CD59). The identity of these cells was not determined. Like P59, the highest expressor (designated E55/59) transmitted its phenotype in a Mendelian fashion, with coinheritance of both transgenes. The ICAM-2 promoter/hCD59 transgenic line 237-7 (dashed line, Fig. 3) showed the strongest expression by flow cytometry (MFI = 644 on granulocytes), with no expression on lymphocytes.

Transgene expression in tissues from heterozygous P59, E55/59 and 237-7 mice was examined by

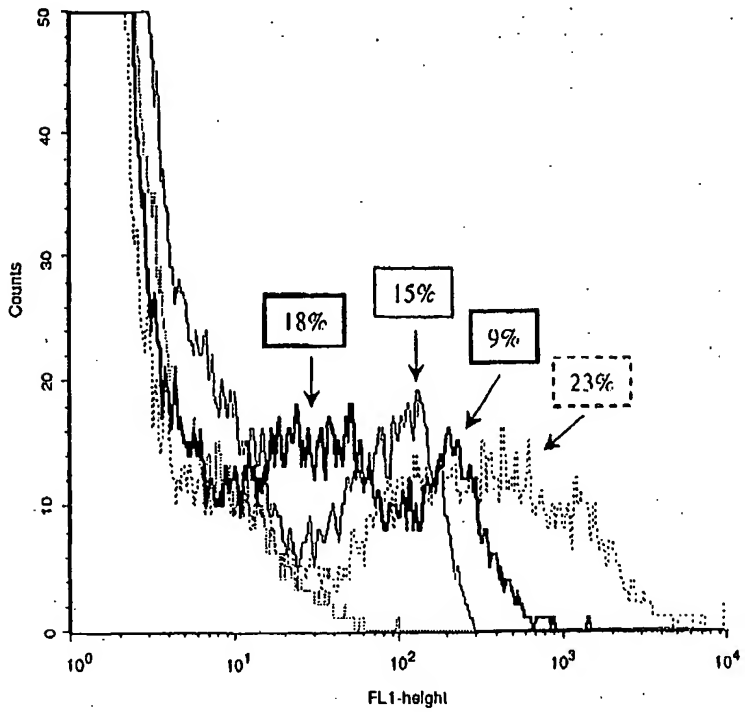

Fig. 3. Flow cytometric analysis of hCD59 expression on peripheral blood leukocytes in heterozygous transgenic mice. Dashed line, 237-7 (ICAM-2 promoter/hCD59); thick line, P59 (PECAM-1 promoter/hCD59); thin line, E55/59 (endoglin promoter/hCD55/hCD59); dotted line, non-transgenic control mouse. Boxed numbers indicate the percentage of total leukocytes within each expressing subpopulation in individual lines.

Table 1. Immunohistochemical staining for human CD59 in tissues from transgenic mice. The promoter used to drive transgene expression in each line is shown in brackets. Key to level of staining: ++++, very strong; +++, strong; ++, moderate, +, weak; -, negative. Note that some tissues in P59 mice contained interspersed areas with varying levels of staining from no staining to strong staining, indicated as -/++++

Organ	Tissue/cell type	237-7 (ICAM-2)	P59 (PECAM-1)	E55/59 (endoglin)
Heart	Medium and large vessels	++++	++++	-
	Microvasculature	++++	-/++++	++++
	Myocytes	-	-	-
Kidney	Medium and large vessels	++++	++++	-
	Glomeruli	++++	-/+++	++++
	Intertubular sinusoids	+++	-/+++	+++
	Tubules	-	-	-
Liver	Central veins	+++	++	+++
	Sinusoidal endothelium	+++	+	++
	Bile ducts	-	-	-
	Hepatocytes	-	-	-
Lung	Medium and large vessels	++++	++++	-/+
	Microvasculature	++++	-/++++	++++
	Bronchioles	-	-	-
Pancreas	All vessels	++++	++++	++++
	Islets	-	-	+
	Duct epithelium	-	-	-

immunohistochemistry. The pattern of expression of hCD55 and hCD59 in tissues from E55/59 mice was identical (data not shown), so for ease of comparison with the other transgenic lines, only results for hCD59 are presented. hCD59 was expressed strongly and uniformly on vascular endothelium in the heart, kidney, liver, lung and pancreas of 237-7 mice (Table 1). Expression driven by the PECAM-1 promoter in P59 mice was also restricted to endothelium but was weaker in some organs, notably the liver, and showed a patchy rather than uniform distribution in the microvasculature of heart, kidney and lung (Table 1). The endoglin promoter (line E55/59) produced a similar pattern and intensity of hCD59 staining to that driven by the ICAM-2 promoter, except that medium and large vessels in heart, kidney and lung were either negative or only weakly positive (Table 1). Weak non-endothelial expression was observed on pancreatic islets in E55/59 mice.

Generation and analysis of triple-transgenic mice and pigs

The ICAM-2 promoter was chosen for the generation of transgenic pigs because it produced the most consistent endothelial-specific expression pattern in transgenic mice. ICAM-2 promoter expression constructs for three hCRPs (hCD46-GPI, hCD55 and hCD59) were mixed in equimolar concentration and used to generate transgenic pigs. As a control the same DNA mixture was used concurrently to generate transgenic mice. Of 340 mice born, 34 (10%) were transgenic and 26 carried more than one transgene. Six of the multitransgenic mice expressed three hCRPs and three mice expressed two hCRPs (Table 2). Expression on leukocytes in different mice ranged from 0.1 to 14

Table 2. Transgene expression on granulocytes in triple-transgenic mice produced by coinjection of ICAM-2 promoter/hCD46-GPI, /hCD55 and /hCD59. Numbers indicate the level of expression relative to the corresponding level on human granulocytes, given an arbitrary value of 1.0

	Transgene expression		
Mouse number	hCD46-GPI	hCD55	hCD59
436	1.2	13.8	4.2
129	0.9	2.6	1.0
167	0.7	1.6	0.7
89	0.6	0.5	1.2
161	0.5	2.3	0.3
127	0.4	0.2	1.9
461	-[a]	2.5	0.3
146	-[a]	1.0	0.2
437	-[a]	0.1	0.1

[a] No detectable expression.

times the corresponding human level and was restricted to granulocytes and monocytes as expected. One mouse (436) expressed all three transgenes at greater than the human level (Table 2). There was no apparent correlation between expression level and transgene copy number, which varied widely (data not shown).

Microinjection into pig oocytes yielded 302 live born piglets, of which 77 (25%) were transgenic (Table 3). The range of copy numbers of each transgene was similar to that observed in the transgenic mice. However, only two of the 65 transgenic pigs weaned expressed hCRPs on leukocytes, and the level of expression was much lower than that seen in the transgenic mice produced with the same DNA. One pig (3544) expressed hCD46-GPI, hCD55 and hCD59 on granulocytes at approximately 10, 20 and 14%, respectively, of the corresponding human level, while the second

Table 3. Transgenic pigs produced by coinjection of ICAM-2 promoter/hCD46-GPI, /hCD55 and /hCD59. Live born piglets were screened to identify transgenics by Southern blot analysis of tail tip DNA. Partial-transgenic indicates that only fragments of the transgene(s) were detected. After weaning, piglets were screened for expression by flow cytometric analysis of peripheral blood leukocytes. Immunohistochemical analysis was performed on (i) liver needle biopsies taken from pigs alive at 3 months old, and (ii) heart and kidney samples taken from several pigs at sacrifice (5 months old)

	Total-transgenic	Triple-transgenic	Double-transgenic	Single-transgenic	Partial-transgenic
Live born (302)	77	33	17	17	10
Weaned (268)	65	29	14	14	8
Positive by flow cytometry		2/29	0/14	0/14	0/8
Positive by liver biopsy		0/23	0/13	0/7	Not done
Positive in heart and/or kidney		2/10	0/5	0/3	Not done

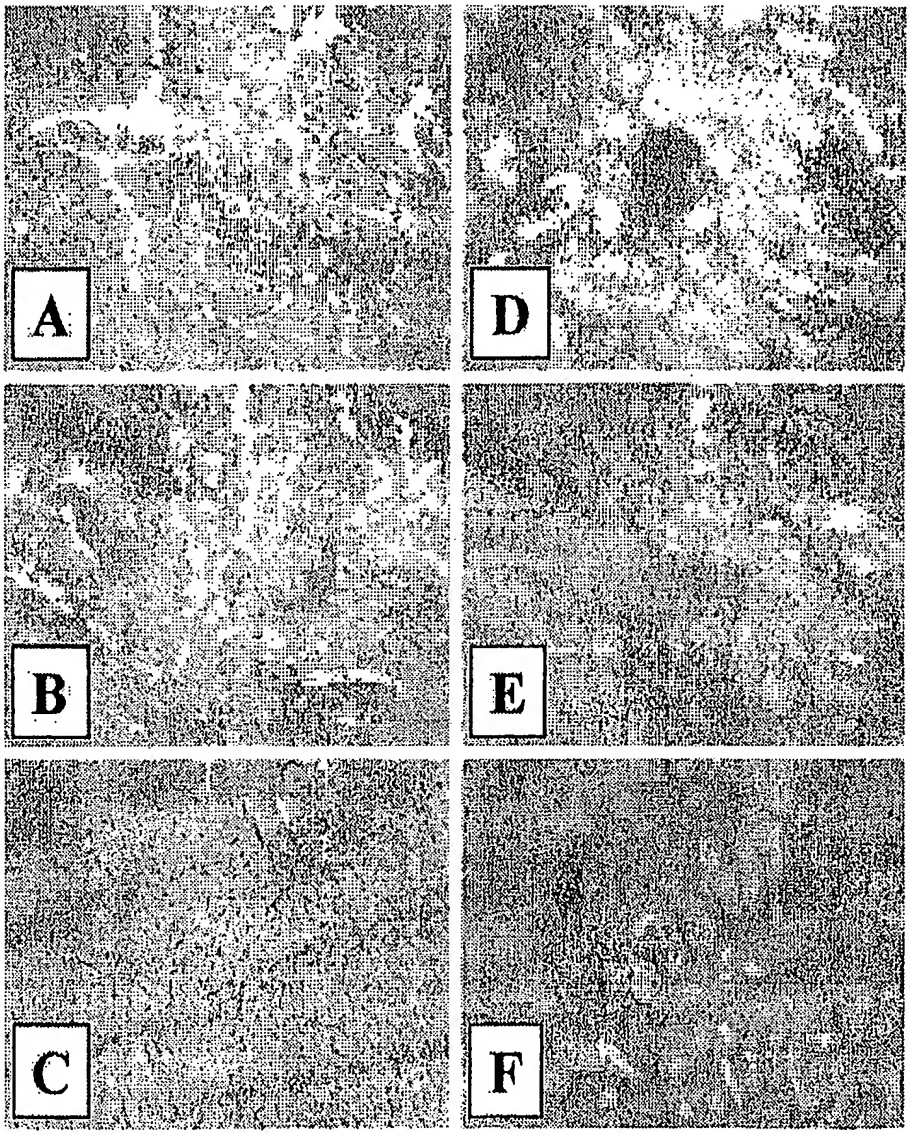

Fig. 4. Immunohistochemical analysis of transgene expression driven by the human ICAM-2 promoter in mice and pigs. *A* and *B*, heart from transgenic pig 3544 stained for hCD55 and hCD59, respectively; *C*, heart from transgenic mouse 436 stained for hCD55. *D* and *E*, kidney from transgenic pig 3544 stained for hCD55 and hCD59, respectively; *F*, kidney from transgenic mouse 436 stained for hCD55.

(3574) expressed hCD55 and hCD59 at less than 10% of the human level (data not shown). To allow for the possibility that expression on leukocytes was not indicative of expression in tissues, liver needle biopsies taken from the 43 surviving pigs that contained at least one intact transgene were examined by immunohistochemistry. No staining was observed on vessels or other tissue in any of the samples. Finally, heart and kidney sections from 18 transgenic pigs were analyzed. Weak endothelial expression of hCD55 and hCD59, but not hCD46-GPI, was detected in pigs 3544 (Fig. 4) and 3574. All other samples were negative.

The low frequency and level of transgene expression in pigs suggested that the 0.33-kb human ICAM-2 promoter functions poorly in pigs compared with mice. Alternatively, pig oocytes may be richer in nucleases than mouse oocytes, leading to greater degradation of the ends of injected DNA fragments prior to genomic integration. In each of the three constructs used to generate transgenic animals, only 40 base pairs separate one end of the DNA fragment from the nearest known functional element of the ICAM-2 promoter [20]. However, polymerase chain reaction analysis of transgene–transgene junctions in 26 pigs revealed minimal degradation of transgene ends, with only one sample containing a deletion extending into the promoter (data not shown).

Discussion

Endothelial targeting is a potentially valuable tool for the transgenic expression of proteins that naturally act only at the endothelial surface, such as human anticoagulants [33], or that may have harmful effects on the donor pig if expressed widely. However, no endothelial-specific promoters have yet been proven in transgenic pigs, perhaps because of the time and expense involved. Measuring the performance of expression constructs in transgenic mice is regarded as the most appropriate and stringent test of promoter activity before proceeding to generate transgenic pigs. Promoters or whole genes that have demonstrated reproducible specificity and activity in transgenic mice and pigs include human CD46, CD55 and CD59 [1,2], mouse H-2K^b [5,6], chicken β-actin [4,34], and pig CD46 [35]. Our results indicate that there may be at least one exception to this general rule. The human ICAM-2 promoter produced tissue-specific hCRP expression, at up to 14-fold the corresponding human level, in 27% of transgenic mice. In contrast, weak transgene expression was detected in less than 4% of pigs containing intact ICAM-2 promoter constructs. This compares unfavorably to frequencies of 20 and 69% expressors in transgenic pigs that we have produced using mouse H-2K^b promoter constructs [36]. Trivial explanations relating to the integrity of the ICAM-2 constructs can be discounted, because the same DNA preparation was used to generate transgenic mice and pigs.

It is conceivable that the low level of expression in transgenic pigs was due to a species-specific effect that was post-transcriptional, such as inefficient mRNA processing (splicing, polyadenylation) or initiation of translation. However, we consider this alternative explanation unlikely, because the signals for these processes in two of the three transgenes (hCD55 and hCD59) were identical to those in H-2K^b-promoter-driven transgenes that have functioned successfully in transgenic pigs [5].

Although it is difficult to draw firm conclusions on the basis of the two weakly expressing pigs, tissue specificity of the human ICAM-2 promoter appeared to be conserved between mice and pigs but the level of activity was not. It is possible that the 0.33-kb promoter fragment used in this study lacked important enhancer(s) necessary for normal activity in pig EC in vivo, and that in the rare examples of detectable expression, enhancer function was provided by endogenous elements near the site of transgene integration. To our knowledge, this is the first reported example of in vivo species specificity of an endothelial-specific or indeed any promoter. On the other hand, in vitro promoter specificity is not without precedent. For example, some regulatory elements were found to differ in behavior and organization in the human and bovine von Willebrand factor promoters [37]. Similarly, analysis of the promoters of the human and mouse P-selectin genes revealed that at least some of the mechanisms of regulation in vitro were species-specific [38]. However, the 0.33-kb human ICAM-2 promoter does not show species specificity in vitro, because similar promoter activity was observed in porcine and bovine EC (Fig. 2). In addition, a 0.36-kb human ICAM-2 promoter fragment was recently reported to be equally active in primary porcine and human aortic EC [39]. This fragment also showed promoter activity in porcine microvascular EC isolated from liver, kidney and brain but not in porcine non-endothelial cells [39].

It is conceivable that ICAM-2 expression is regulated differently in pigs than in humans and mice. The related adhesion molecule ICAM-1 shows some differences in basal expression and up-regulation in porcine and human cells [40]. Nevertheless we consider this possibility unlikely because ICAM-2 is

constitutively expressed in a similar predominantly endothelial-restricted pattern in humans and mice [41], probably because of its essential role in the trafficking of eosinophils [42] and possibly dendritic cells [43]. We also have preliminary evidence that ICAM-2 is highly expressed in cultured porcine EC and in endothelium-rich porcine tissues (data not shown). As a logical next step, we are investigating the use of regulatory elements from the pig ICAM-2 gene to express transgenes.

This report contains the first description of transgene expression in vivo driven by human PECAM-1 and endoglin promoter fragments. Despite its lack of specificity in vitro, the PECAM-1 promoter directed endothelial-specific expression in transgenic mice, but produced an unusual pattern in which only a proportion of blood vessels within various organs expressed the transgene. This was surprising because PECAM-1's native expression pattern in the vasculature is uniform to the extent that it is commonly used as a pan-endothelial marker. The transgene expression pattern was unlikely to be an aberration caused by integration site effects or mosaicism, because it was observed in independently generated transgenic mice and was transmissible with normal Mendelian inheritance. The endoglin promoter also showed non-uniform endothelial-specific transgene expression, with strong staining of small but not large vessels in several organs. This is consistent with several reports suggesting preferential expression of endoglin in the microvasculature [44–46]. The endoglin promoter may be useful in xenotransplantation because the triggering events of xenograft rejection appear to be focussed in the microvasculature, particularly on the venous side [47]. However, in the light of the poor result with the ICAM-2 promoter, it is clear that although transgenic mice provide some information regarding promoter activity in vivo, making transgenic pigs is the only certain way of assessing candidate tissue-specific promoters.

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Spontaneous Inflammatory Disease in Transgenic Rats Expressing HLA-B27 and Human β_2m: An Animal Model of HLA-B27-Associated Human Disorders

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Summary

Humans who have inherited the human class I major histocompatibility allele HLA-B27 have a markedly increased risk of developing the multi-organ system diseases termed spondyloarthropathies. To investigate the role of B27 in these disorders, we introduced the B27 and human β_2-microglobulin genes into rats, a species known to be quite susceptible to experimentally induced inflammatory disease. Rats from one transgenic line spontaneously developed inflammatory disease involving the gastrointestinal tract, peripheral and vertebral joints, male genital tract, skin, nails, and heart. This pattern of organ system involvement showed a striking resemblance to the B27-associated human disorders. These results establish that B27 plays a central role in the pathogenesis of the multi-organ system processes of the spondyloarthropathies. Elucidation of the role of B27 should be facilitated by this transgenic model.

Introduction

Class I major histocompatibility (MHC) gene products are polymorphic 44,000 M_r glycoproteins expressed on cell surfaces in noncovalent association with the nonpolymorphic 12,000 M_r light chain β_2-microglobulin (Klein, 1986). Among class I MHC molecules, HLA-B27, a serologically defined allele of the human *HLA-B* locus, is of particular interest because it is uniquely associated with a group of relatively common inflammatory disorders. The strongest association is seen with primary ankylosing spondylitis, a chronic inflammatory disease affecting the axial musculoskeletal system: ~90% of affected individuals have inherited the B27 allele in comparison with only ~7% of Caucasians in the general population (Brewerton et al., 1973; Schlosstein et al., 1973; Tiwari and Terasaki, 1985). An important association also exists between HLA-B27 and reactive arthritis, in which certain microbial infections of the gastrointestinal or genitourinary tracts trigger inflammation in joints and other tissues (Toivanen and Toivanen, 1988). A summary of the major disorders associated with HLA-B27 is presented in Table 1.

The B27-associated diseases are classified as rheumatic disorders because of the prominence of musculoskeletal manifestations. Nonetheless, all of these diseases can involve multiple organ systems, particularly the gastrointestinal tract, genitourinary tract, skin, eye, and heart. Because of the overlap among these diseases with regard to epidemiology, clinical manifestations, and anatomic pathology, they were recognized as a distinct cluster of interrelated diseases, termed spondyloarthropathies, even before the common genetic marker of B27 was identified (Moll et al., 1974). Thus it has long been speculated that a common pathogenetic mechanism might underlie the association of B27 with this heterogeneous group of disorders. Despite extensive investigation, however, the etiology and pathogenesis of these diseases have remained obscure, and the basis for the association with B27 has not been established.

In an attempt to develop an animal model of B27-associated disease, we (Taurog et al., 1988a) and others (Krimpenfort et al., 1987; Nickerson et al., 1990; Weiss et al., 1990) have produced transgenic mice expressing HLA-B27 and human β_2-microglobulin (hβ_2m). However, despite physiologically normal function of B27 in both hybrid and inbred mice (Kievits et al., 1987; Taurog et al., 1988a) and a reported influence of B27 on the course of an experimental bacterial infection in mice (Nickerson et al., 1990), no faithful reproduction of any of the features of B27-associated human disease has been reported in transgenic mice. These negative results raised the possibility that susceptibility to the spondyloarthropathies might not be related to the B27 gene. Alternatively, other features of the mouse may not have permitted expression of the relevant pathologic changes. We therefore sought to develop transgenic technology in rats, which are susceptible to several experimentally induced arthritic diseases that cannot be elicited in mice (Greenwald and Diamond, 1988).

In this paper, we describe the production of transgenic rats that express HLA-B27 and hβ_2m genes. We further describe a disorder spontaneously arising in these B27 transgenic rats that includes most of the features of B27-associated disease in humans.

Results

Integration of HLA-B27 and hβ_2m Genes in Inbred Rats

Fertilized one-cell rat eggs were microinjected with a solution containing both DNA fragments shown in Figure 1. The HLA-B27 gene encoding the HLA-B*2705 subtype was contained on a 6.5 kb EcoRI fragment that included 0.7 kb of 5′ flanking sequence and 2.5 kb of 3′ flanking sequence (Figure 1A). The hβ_2m gene was contained on a 15 kb SalI–PvuI fragment that included 5.2 kb of 5′ flanking sequence and 1.9 kb of 3′ flanking sequence (Figure 1B). Identification and quantitation of transgenes in the founder animals and their progeny were determined by dot-blot hybridization of genomic DNA isolated from tail bi-

Table 1. Rheumatic Diseases Associated with HLA-B27

Characteristic	Disorder				
	Ankylosing Spondylitis	Reactive Arthritis[a]	Juvenile Spondyloarthropathy	Psoriatic Arthropathy	Enteropathic Arthropathy
Sacroiliitis or spondylitis[b]	100%	<50%	<50%	20%	10%
Peripheral arthritis[c]	25%	90%	90%	95%	90%
Gastrointestinal inflammation	Common, usually asymptomatic	Common, often symptomatic	Not known	Uncommon	All
Skin and nail involvement	Rare	Most	Uncommon	All	Uncommon
Genitourinary involvement (males only)	Uncommon	Most	Uncommon	Uncommon	Rare
Eye involvement[d]	25%	Common	Common	Occasional	Occasional
Cardiac involvement	<5%	5%–10%	Not known, probably rare	Rare	Rare
Usual age of onset (years)	18–40	18–45	7–18	20–50	15–50
Sex prevalence	Males 3:1	Males 3:1[e]	Males 10:1	Equal	Equal
Type of onset	Gradual	Acute	Variable	Variable	Gradual
Role of infectious agents	Unknown	Definite Trigger	Unknown	Unknown	Unknown
Prevalence of HLA-B27[f]	>90%	60%–80%	80%	50%[g]	50%–75%[g]

Table adapted from Calin (1984); Tiwari and Terasaki (1985); Khan and van der Linden (1990); Taurog and Lipsky (1990).
[a] Includes Reiter's syndrome, classically defined as the triad of arthritis, conjunctivitis, and urethritis.
[b] Inflammation in the spine or sacroiliac joints.
[c] Inflammation in joints of the extremities.
[d] Predominantly conjunctivitis in reactive arthritis; iritis with the other disorders.
[e] Male to female ratio is 10:1 if venereally acquired; 1:1 if enteropathically acquired.
[f] Caucasians of northern European extraction only. General prevalence in this population is 6%–8%. Some variation seen in other populations, but the basic associations with HLA-B27 are seen worldwide.
[g] Frequency elevated only in those with spondylitis or sacroiliitis.

opsies. Hybridization was carried out with 5′ and 3′ flanking probes for the HLA-B27 gene (probes A and C in Figure 1A), and with a 3.7 kb BglII fragment containing exons 2 and 3 of the $h\beta_2m$ gene (probe D in Figure 1B).

Seven LEW and four F344 rats that developed from microinjected ova showed integration of the HLA-B27 and $h\beta_2m$ genes. Of these, four LEW rats and one F344 rat showed cell surface expression of both HLA-B27 and $h\beta_2m$, as assessed by indirect immunofluorescence of peripheral blood lymphocytes (PBLs). One additional LEW rat showed integration and expression of the B27 gene alone. Table 2 summarizes the results of the microinjection experiments.

All of the founder rats expressing the transgenes were subsequently shown to transmit the transgenes to their offspring. One of the six founders, 21-3, was found to be a mosaic, based on non-Mendelian rates of transmission and on enhanced cell surface expression in the offspring. Another founder, 21-4, a female, was shown to have two independently segregating loci of transgene integration, each locus carrying both transgenes. One line arising from this founder, inheriting a locus containing 150 copies of the B27 gene and 90 copies of the $h\beta_2m$ gene, was termed 21-4H. The other line, inheriting a locus containing six copies of the B27 gene and six copies of the $h\beta_2m$ gene, was termed 21-4L (Table 3).

Lymphocyte Cell Surface Expression of the HLA-B27 and $h\beta_2m$ Transgene Products

Expression of the transgene products was estimated by indirect immunofluorescence and flow cytometry of PBLs stained with specific monoclonal antibodies. The relative expression of B27 and $h\beta_2m$ in seven transgenic lines is shown in Table 3. To compensate for interexperiment variation, the mean channel fluorescence for each line with each antibody is expressed relative to that determined in the same experiment for PBLs of the transgenic mouse line 56-3, which expresses high levels of both B27 and $h\beta_2m$ on PBL surfaces. The highest expression of both gene products was found in the LEW lines 21-4H and 21-4L and the F344 line 33-3.

The patterns of cell surface expression of B27 and $h\beta_2m$ in the 21-4H and 21-4L lines are shown in Figures 2A and 2B. The binding of the endogenous rat class MHC I molecules (RT1) to the anti-RT1 antibody OX18 is shown in Figure 2C for both transgenic lines and the nontransgenic control. The levels of expression of B27 and $h\beta_2m$ were comparable in the two transgenic lines (Figures 2A

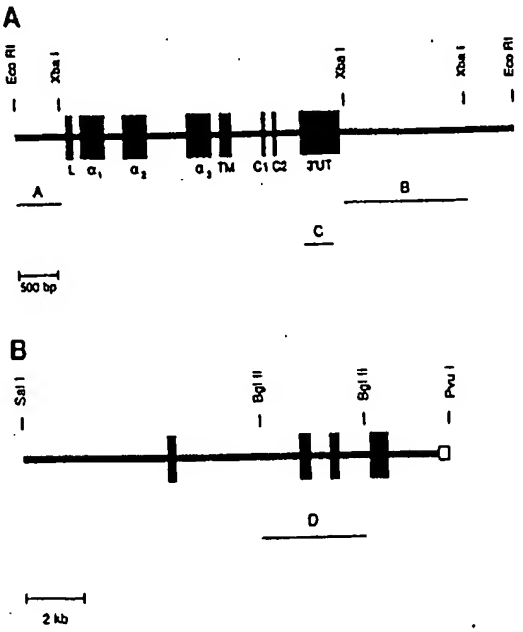

Figure 1. Genes Used for Microinjection of Fertilized Rat Eggs

(A) The HLA-B*2705 gene (clone pE.1-B27) was contained on a 6.5 kb EcoRI fragment. Exons are indicated by boxes and labeled. Probes from the 5' and 3' flanking regions, labeled A and B, respectively, were used for dot-blot hybridization of genomic DNA. Probe C, from the 3' untranslated region, was used for Northern hybridization.

(B) The hβ2m gene (clone pβ2m-13) was contained on a 15 kb SalI–PvuI fragment. Exons are indicated by boxes. The insert contained ~100 bp of the vector pEMBL9, indicated by the open box at the 3' end. The 3.7 kb BglII fragment labeled D was used for both dot-blot hybridization of genomic DNA and for Northern hybridization.

Table 2. Production of HLA-B27 and hβ2m Transgenic Rats

			Founder			
			Integration[b]		Expression[c]	
Strain	Eggs[a]	Pups	B27	hβ2m	B27	hβ2m
LEW	348	23	8	7	5	4
F344	329	24	4	4	1	1

[a] Number of eggs injected and transferred to pseudopregnant recipients.

[b] Transgenic animals were identified by dot-blot analysis of DNA isolated from tails.

[c] Cell surface expression was assessed by indirect immunofluorescence and flow cytometry of PBLs.

Table 3. Copy Number and Cell Surface Expression of HLA-B27 and hβ2m in Transgenic Rat Lines

	Gene (Copy/Cell)[a]		Cell Surface Expression (Relative MCF)[b]	
Line	B27	hβ2m	B27	hβ2m
21-2	1	1	0.09	0.06
21-3	20	15	0.30	0.29
21-4L	6	6	0.74	0.42
21-4H	150	90	0.51	0.42
25-1	1	0	0.15	0.00
25-6	7	7	0.42	0.27
33-3	55	66	1.00	0.76

[a] Gene copy number was estimated by quantitative dot hybridization on DNA isolated from tails using probes specific for each transgene (see Figure 1A).

[b] Mean channel fluorescence (MCF) with antibodies to HLA-B (B1.23.2) or hβ2m (BBM.1) of PBLs from transgenic rats, relative to simultaneously determined MCF of PBLs from the B27/hβ2m transgenic mouse line 56-3. All data are from progeny of founders to eliminate influence of mosaicism.

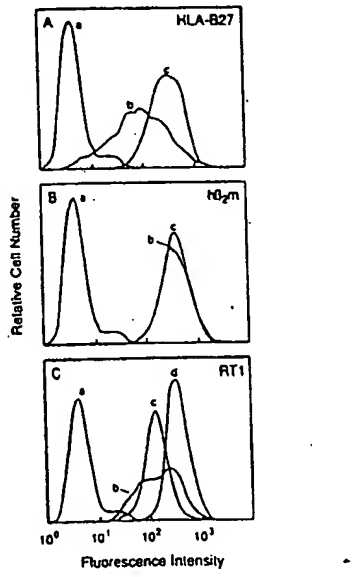

Figure 2. Comparison of Cell Surface Expression of HLA-B27, hβ2m, and the Endogenous RT1 Class I MHC Molecules in 21-4H, 21-4L, and Nontransgenic Rats

Peripheral blood mononuclear cells were incubated with saturating concentrations of monoclonal antibodies and fluorescein-labeled second antibodies and then analyzed by flow cytometry, as described in Experimental Procedures. The results demonstrate that cell surface expression of both transgenes was at least as high in the clinically normal 21-4L line as in the disease-prone 21-4H line and that endogenous RT1 expression appeared lower in the transgenic rats than in the nontransgenic control. Sources of cell populations were nontransgenic LEW stained with negative control antibody (a), 21-4H (b), 21-4L (c), and nontransgenic LEW stained with anti-RT1 antibody (d). Monoclonal antibodies were anti-HLA-B27 (B.1.23.2) (A), anti-hβ2m (BBM.1) (B), and anti-RT1 class I (OX18) (C).

and 2B), and in both lines the expression of the endogenous RT1 class I molecules appeared to be reduced in comparison with the nontransgenic control (Figure 2C).

Immunologic Function of the HLA-B27 Transgene

To assess T cell recognition of the B27 transgene product as a class I MHC antigen, primary grafts of B27 transgenic LEW rat skin were placed on nontransgenic LEW rats, and spleen cells from the recipient rats were subsequently tested for B27-specific cytotoxicity. As shown in Table 4,

Table 4. Cell-Mediated Cytotoxicity against HLA-B27

Effector Cells		Effector to	% Cytoxicity of Target Cells	
Donor	Recipient	Target Ratio	$B27^+h\beta_2m^+$	$B27^-h\beta_2m^+$
Experiment 1				
21-4L	LEW	100	34	15
		50	38	8
		20	36	3
LEW	LEW	100	19	13
		50	14	7
		20	5	1
Experiment 2				
21-4H	LEW	100	18	7
		50	8	4
		20	3	2
LEW	LEW	100	3	3
		50	1	2
		20	0	1

Spleen cells from LEW rats grafted 7 days earlier with skin from either 21-4 transgenic or normal LEW donors were incubated at the indicated effector target ratios with ^{51}Cr-labeled murine L cell targets expressing either $h\beta_2m$ alone or $h\beta_2m$ and HLA-B27. Incubation times: experiment 1, 6 hr; experiment 2, 4 hr. SD <15% in experiment 1 and <10% in experiment 2.

spleen cells from nontransgenic LEW rats receiving grafts from either 21-4H or 21-4L donors showed significantly higher lytic activity against L cell targets transfected with the B27 gene than against otherwise identical targets lacking this gene. Lytic activity was also higher in recipients of transgenic grafts than in recipients of control nontransgenic syngeneic grafts. These results indicate that the B27 transgene product is recognized in a conventional manner by allogeneically primed cytolytic T cells.

Inflammatory Disease in the 21-4H Line: Clinical and Histologic Findings

Gastrointestinal Tract

Overt disease appeared in all of the rats bearing the 21-4H transgene locus that survived past 10 weeks of age. This cohort consisted of 14 males and 9 females. The most common and persistent finding was diarrhea, manifested by frequent, voluminous, often watery stools. Diarrhea was observed in all 23 animals, with equal persistence and severity in the two sexes. Histologically, the gastrointestinal disease was manifested by chronic inflammation involving the stomach and small and large intestine (Figure 3). The distribution and severity of the lesions varied, the colon being the most consistently and prominently affected site. Less frequently, gastric lesions predominated. In all sites, the inflammatory cells consisted primarily of large and small lymphocytes, plasma cells, and smaller numbers of eosinophils. Although the inflammatory response remained primarily in the lamina propria, in the most severely affected regions it extended into the submucosa. Lymphocytes were commonly aggregated into small hyperplastic lymphoid foci, especially in the colon and ileum.

In the intestinal lesions, hyperplasia of crypt epithelial cells replaced mucus-secreting cells and increased the depth of the crypts (Figures 3D and 3F). Hyperplastic crypt cells showed regenerative atypia and a marked increase in mitotic activity. Destruction of crypts and/or the formation of crypt abscesses was uncommon and seen only in the most inflamed areas.

The gastric lesions generally consisted of widely scattered inflammatory foci in the lamina propria and submucosa, but in more severe lesions inflammation was much more extensive, and inflammatory cells accumulated in ectatic glands. The proliferation of mucus-neck cells resulted in marked reduction in the number of parietal cells (Figure 3B).

That the gastrointestinal inflammation did not result from a contagious pathogen was suggested by four pieces of evidence. Stool cultures for aerobic bacteria yielded only normal fecal flora. Furthermore, rats of the 21-4L line and nontransgenic LEW rats were housed for long periods in the same cages with affected 21-4H rats without showing any diarrhea or other signs of illness. In addition, the histology of the gastrointestinal tract of the affected 21-4H rats was not consistent with any known infectious process. Finally, diarrhea has also appeared in six out of seven transgenic rats of the 33-3 line past the age of 2 months, and not in their nontransgenic littermates.

Peripheral and Axial Joints

Peripheral arthritis was observed in 10 of 14 21-4H males and in 1 of 9 21-4H females. This was manifested in most cases by swelling, erythema, and tenderness of the tarsal joints of one or both hindlimbs (Figure 4B). In a few animals the carpal joints or digits were also inflamed (Figure 4D). The arthritis persisted from a few days to several weeks, and in some cases showed an undulating pattern of remission and exacerbation.

Histologically, large accumulations of neutrophils were present in the joint space. The synovium was hyperplastic, edematous and infiltrated with large numbers of lymphocytes, plasma cells, and neutrophils, with neutrophils predominating in the most active lesions (Figure 6B). There was marked pannus formation that eroded the bone at the synovial recess, invading and destroying the articular cartilage. Where the articular cartilage on adjacent joint surfaces was completely replaced by pannus, fibrous ankylosis occurred. Reactive bone formed small osteophytes along the diaphyses, and foci of metaplastic bone were seen within the fibrotic joint capsule. Chronic inflammation extended from the joint capsule to involve adjacent ligaments and tendons. Despite extensive joint destruction evident histologically, resolution generally occurred with preservation of mobility in the large joints.

Vertebral joints from two tails of 21-4H rats were examined histologically, and both revealed inflammatory changes at the outer aspects of the annulus fibrosus and its attachment to the vertebral endplate (Figure 6D). The inflammatory cells consisted of lymphocytes and small numbers of plasma cells mixed with active fibroblasts. There was active bone resorption at the insertion of the annulus and the adjacent periosteum was reactive.

Skin and Nails

Several animals of both sexes developed grossly evident

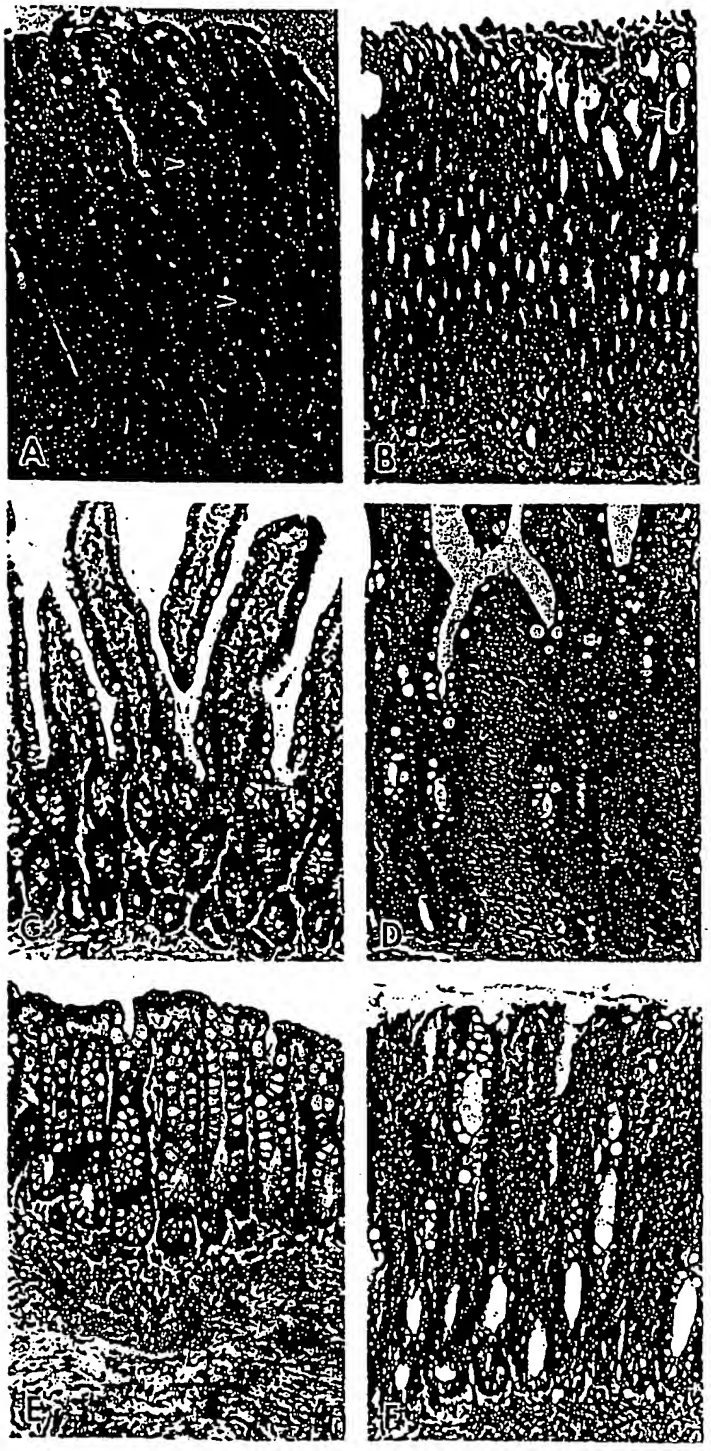

Figure 3. Gastrointestinal Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.
(A) Normal stomach. Arrowheads indicate typical parietal cells (81.25×).
(B) Stomach of a 3-month-old 21-4H male, showing chronic gastritis, with numerous dilated pits and glands (asterisks). A microabscess is present in one dilated gland (arrowhead). Hyperplasia of the mucus-neck cells has largely replaced the parietal cells, and an inflammatory infiltrate is present throughout the lamina propria (65×).
(C) Normal ileum (84.5×).
(D) Ileum of a 3-month-old 21-4H male, showing chronic enteritis. The depth of the crypts is increased due to epithelial cell hyperplasia. There is a loss of mucus-secreting cells, and an inflammatory infiltrate is present throughout the lamina propria (84.5×).
(E) Normal colon (97.5×).
(F) Colon of a 3-month-old 21-4H male, showing chronic colitis. The depth of the crypts is markedly increased due to epithelial cell hyperplasia. There is a loss of mucus-secreting cells, and an inflammatory infiltrate is present throughout the lamina propria (97.5×).

changes in the tail skin and/or dramatic hyperkeratosis and dystrophy of the nails on all four extremities (Figures 5B and 5D). Histologically, in the tail lesions the epidermis was massively thickened by psoriasiform hyperplasia (Figure 6F). The rete ridges were regular and thickened at the base. Exocytosis of lymphocytes and neutrophils was common, with these cells accumulating in spongiotic foci in the epidermis, in the superficial parakeratotic crust, or around degenerated, necrotic keratinocytes. Diffuse orthokeratotic hyperkeratosis was prominent. The superfi-

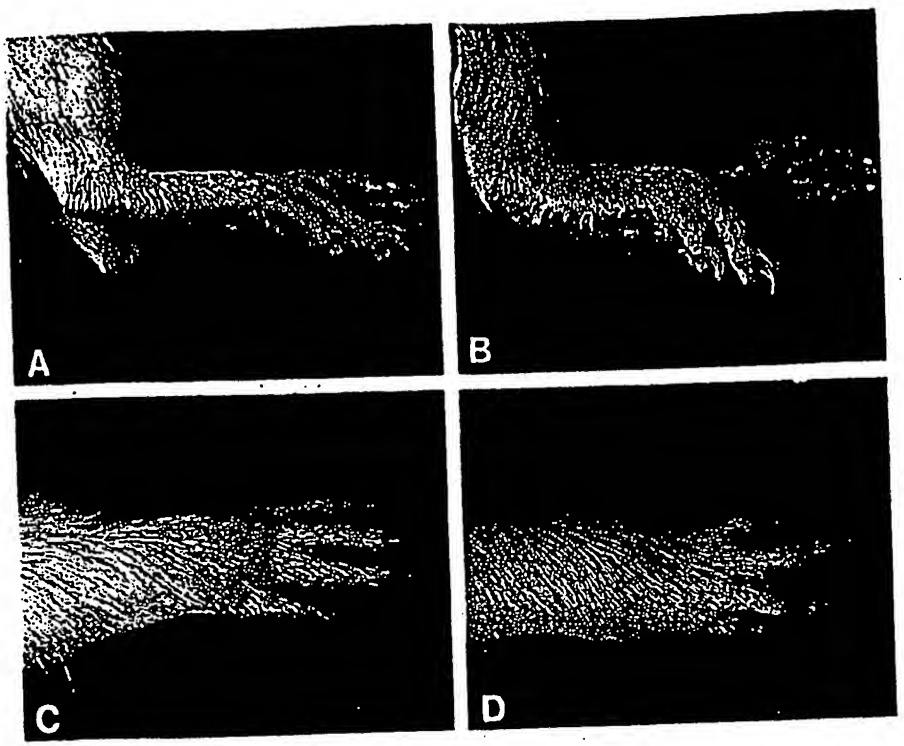

Figure 4. Peripheral Joint Gross Pathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.
(A) Normal distal hindlimb.
(B) Distal hindlimb of a 6-month-old 21-4H male showing swelling and erythema.
(C) Normal distal forelimb.
(D) Distal forelimb of a 4-month-old 21-4H male showing swelling and erythema surrounding the carpal joint.

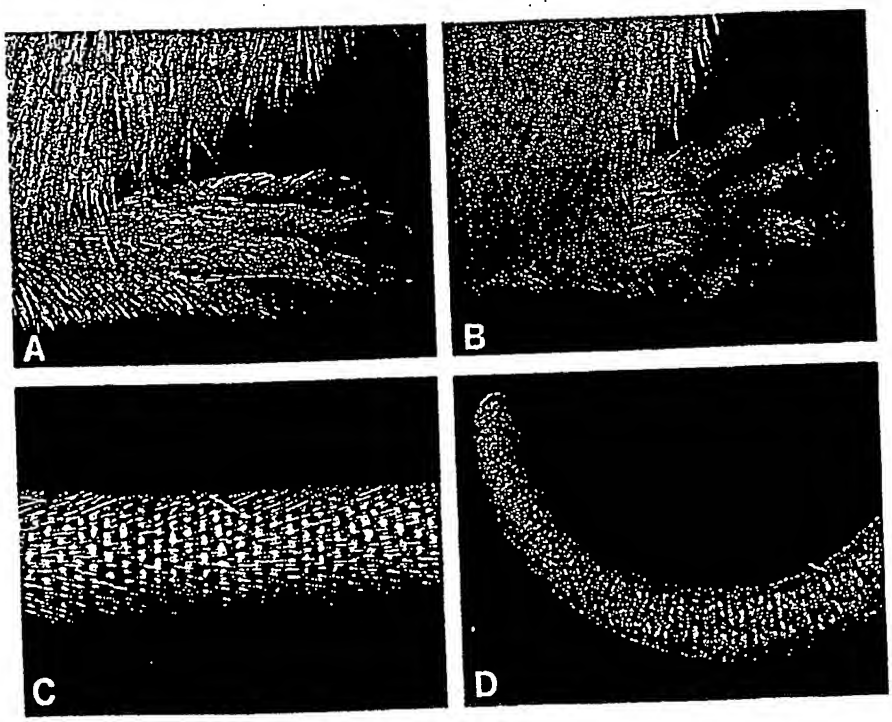

Figure 5. Nail and Skin Gross Pathology of 21-4H Rats

(A) Normal hindlimb digits and nails.
(B) Hindlimb digits and nails of a 3½-month-old 21-4 male, showing hyperkeratosis and dystrophy of the nails and alopecia over the digits.
(C) Normal tail.
(D) Tail of a 3½-month-old 21-4 male (same as in [B]), showing edema, alopecia, flaking, and masking of the normal ridged pattern.

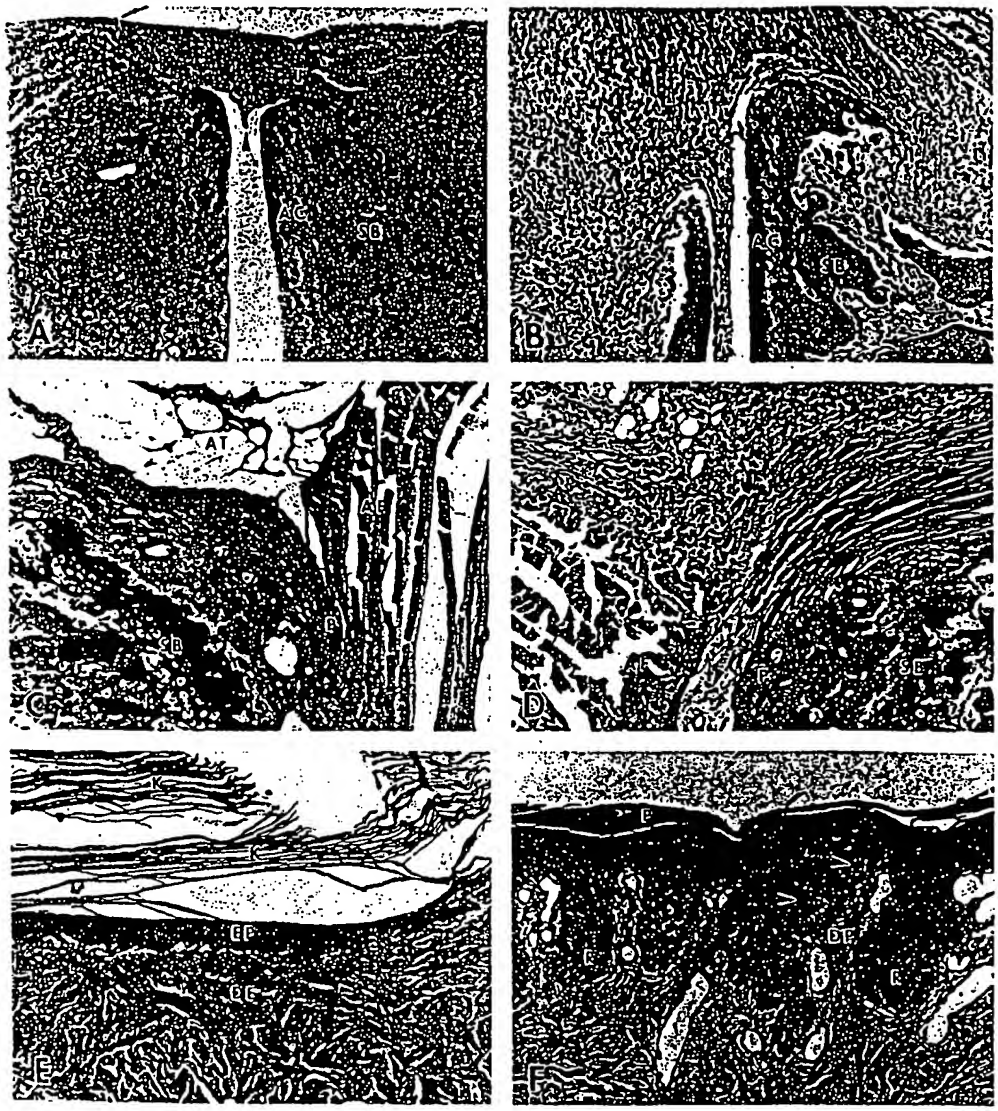

Figure 6. Peripheral and Axial Joint and Skin Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.

(A) Normal tarsal joint. Synovium (arrowhead), articular cartilage (AC), subchondral bone (SB), and joint capsule (J) are labeled (78×).

(B) Tarsal joint of a 4-month-old 21-4H male (same as in Figure 4D), showing chronic arthritis. There is a marked inflammatory infiltrate in the joint capsule and synovium, with pannus (asterisks) eroding articular cartilage (AC) and subchondral bone (SB) on both sides of the joint (58.5×).

(C) Normal tail intervertebral joint. The annulus fibrosus (AF), vertebral end plate (P), ossification center of subchondral bone (SB), and periarticular adipose tissue (AT) are labeled (65×).

(D) Tail intervertebral joint of a 4-month-old 21-4H male (same as in Figure 4D), oriented as a mirror image of (C), showing expansion of the periarticular connective tissue by mononuclear inflammation and fibrosis (asterisks), invading and disrupting the attachment of the outer layers of the annulus to the vertebral end plate (arrowheads). Annulus fibrosus, vertebral endplate, and subchondral bone are labeled as in (C) (58.5×).

(E) Normal tail skin. The keratin layer (K) overlies the epidermis (EP) and dermis (DE) (97.5×).

(F) Tail skin of a 3½-month-old 21-4 male (same as in Figure 5B), showing prominent, elongate, regular rete pegs (R) (psoriasiform epidermal hyperplasia), exocytosis of lymphocytes and neutrophils (arrowheads), parakeratosis (P), and dermal papillae (DP) containing inflammatory infiltrates (78×).

cial papillary dermis contained a diffuse infiltrate of neutrophils, lymphocytes, and plasma cells. Similar changes were seen in skin over the distal aspect of the digits.

Testis and Epididymis

Orchitis and epididymitis were prominent findings in the 21-4H males. The orchitis was manifested clinically by a progressive enlargement of the testes followed by testicular atrophy, with infertility ensuing by 3 months of age in most of the males. In contrast, the females showed little loss of fertility, even in the presence of persistent diarrhea. Histologically, the testicular tunica was thickened by connective tissue, which contained active angioblasts and fi-

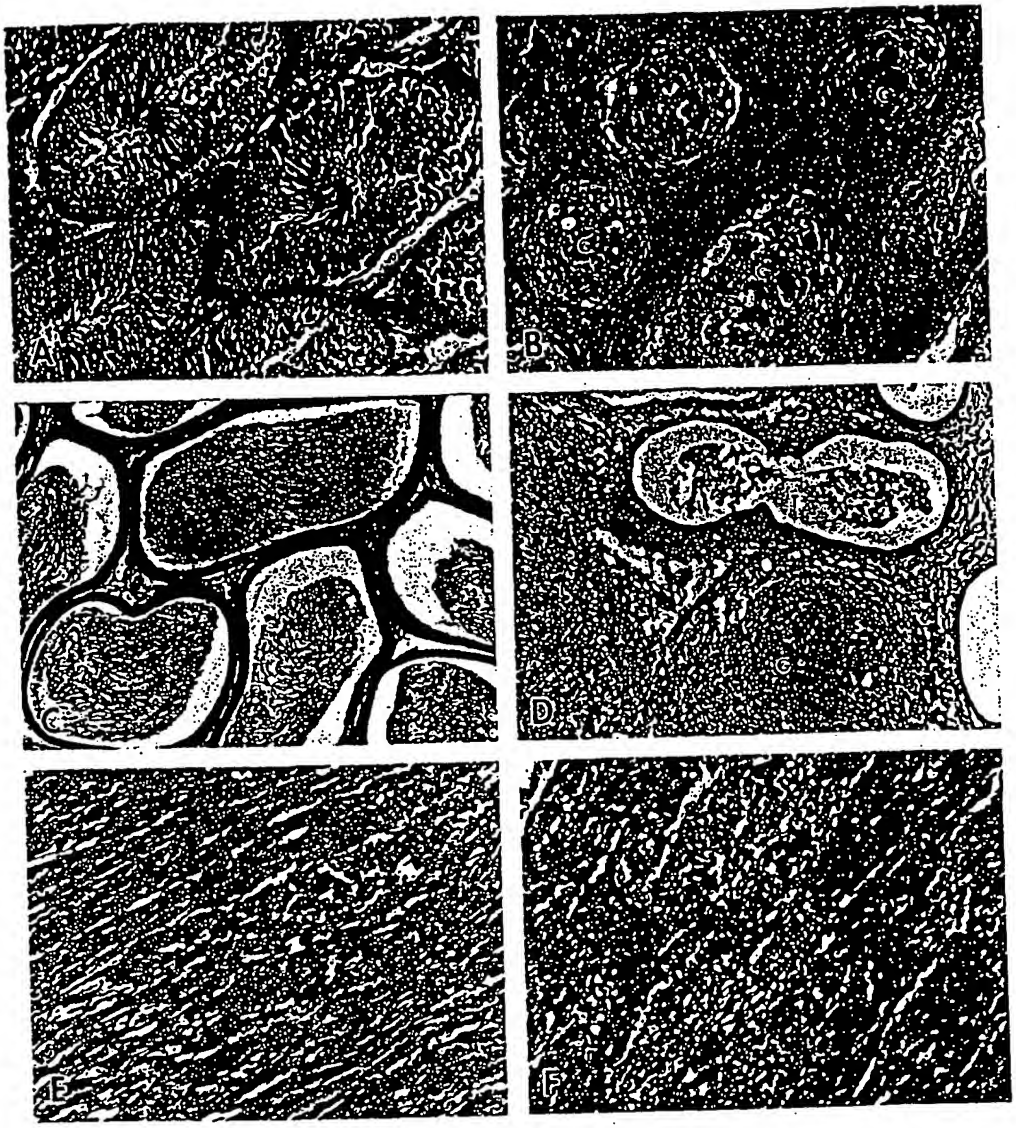

Figure 7. Male Genital Tract and Myocardial Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.

(A) Normal testis (71.5×).

(B) Testis of a 3-month-old 21-4H male, showing chronic orchitis with an intense mononuclear cell interstitial inflammatory infiltrate and sperm granulomas (G) (71.5×).

(C) Normal epididymis (65×).

(D) Epididymis of a 3-month-old 21-4H male, showing chronic epididymitis with a granulomatous interstitial inflammatory cell infiltrate (I), sperm granuloma (G), and dilated tubules (T) containing degenerated inflammatory cells and no sperm (65×).

(E) Normal myocardium (78×).

(F) Myocardium of a 3-month-old 21-4H male (same as in Figure 3F), showing myocarditis with a prominent mononuclear inflammatory cell infiltrate separating the myofibers (78×).

broblasts as well as large numbers of lymphocytes and plasma cells. The testes often contained numerous granulomas with necrotic centers surrounded by epithelioid macrophages and giant cells and peripherally by lymphocytes, plasma cells, and fibrosis (Figure 7B). Central infarction of the testis was a common finding in the most severely affected specimens.

The epididymis frequently contained granulomas similar to those found in the testis, along with dilated tubules containing necrotic cellular debris. The interstitium of the epididymis was expanded by lymphocytes, plasma cells, epithelioid macrophages, and moderate fibrosis (Figure 7D).

Heart

Active inflammatory lesions were evident histologically in four of nine 21-4H hearts examined (Figure 7F). In one specimen, extensive multifocal lesions were seen, involving the ventricular walls and septum. The lesions con-

sisted of large numbers of lymphocytes and small numbers of plasma cells, macrophages, and eosinophils. The myofibers were widely separated by the inflammatory cells, and scattered karyorrhectic nuclei were seen. In the less severely affected specimens, infiltrates of lymphocytes and plasma cells were found at the root of the aortic valve. In more chronic lesions there was moderate fibrosis scattered throughout the myocardium accompanied by mild lymphocytic inflammation. In one animal the adventitia of the great vessels was infiltrated by large numbers of lymphocytes and plasma cells admixed with proliferating angioblasts and fibroblasts.

Eye and Central Nervous System

Mild keratitis and anterior uveitis were observed histologically in one of five eyes from 21-4H rats, one of five eyes from 21-4L rats, and none of four eyes from nontransgenic LEW rats. These findings were judged to be nonspecific, probably secondary to bacterial keratitis.

A peculiar neurologic syndrome was seen in all of the females and most of the males of the 21-4H line. This was manifested by cerebellar ataxia, with intermittent episodes of a stereotypical muscular dystonia, usually in response to handling or some other mild stimulus. Electrophysiologic studies during these episodes demonstrated increased muscular tone without evidence of a cortical seizure focus (data not shown). For several reasons, this abnormality was thought to result from a process distinct from that giving rise to the other lesions. Whereas the other lesions appeared after puberty and then progressed, the neurologic abnormality began within a few weeks after birth and showed no increase in severity thereafter. Unlike the other disease processes, the clinical pattern of the neurologic findings showed little variation from rat to rat. Furthermore, the histologic abnormalities associated with the neurologic disease, which involved primarily the spinal cord and cerebellum, were not inflammatory (data not shown). Finally, there was no evidence of neurologic disturbance in the transgenic F344 line that also showed diarrhea, nor in any of the other transgenic LEW lines.

Other Tissues

The following tissues were examined in at least one of the 21-4H rats showing diarrhea and found not to show histologic abnormalities: esophagus, lung, liver, kidney, adrenal, pancreas, penis, spleen, and thymus. Atrophy of thymus and spleen that was apparent to gross examination was a common finding, however, along with peripheral and mesenteric lymph node enlargement.

Clinical and Histologic Findings in Other Transgenic Lines

No clinical abnormalities were noted in any of the B27 transgenic LEW lines other than 21-4H. Histologic tissue surveys of several 21-4L rats revealed a mild degree of intestinal lymphoid hyperplasia and fibrosis as the only abnormality. Similar intestinal lesions were also found at a lower frequency in nontransgenic controls, and hence the significance of these findings in the 21-4L rats is not yet established. As noted above, almost all transgenic rats of the F344 line 33-3 showed diarrhea by 2 months of age.

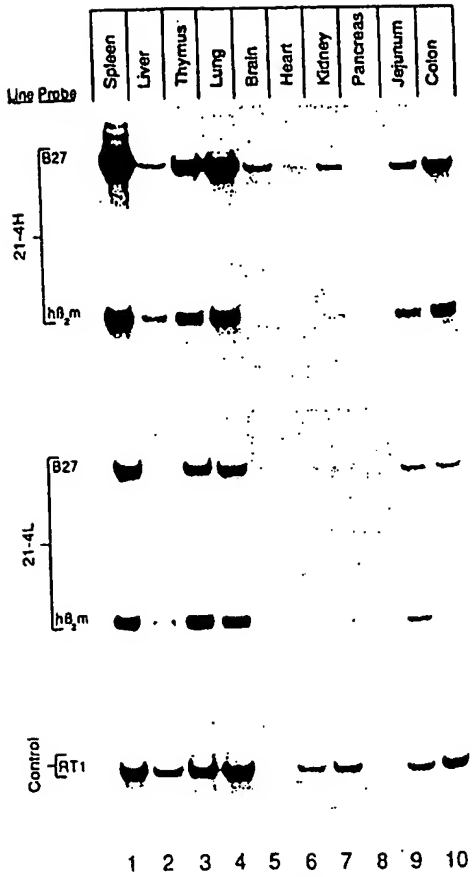

Figure 8. Northern Blot Analysis of HLA-B27, $h\beta_2m$, and RT1 mRNA: Tissue Survey

Total cellular RNA from tissues of 12-week-old male 21-4H, 21-4L, and nontransgenic control rats was subjected to denaturing agarose gel electrophoresis (10 μg per lane), transferred to nylon membranes, and hybridized to ^{32}P-labeled probes as described in Experimental Procedures and Figure 1. Membranes were exposed to XAR-5 film at −70°C with intensifying screens for 2–26 hr.

Tissue Distribution of mRNA Expression

Despite the striking differences in disease manifestations, the 21-4H and 21-4L lines showed similar cell surface expression of the transgene products in PBLs (Table 3; Figures 2A and 2B). It was thus of interest to compare the two lines with respect to the level and tissue distribution of mRNA transcripts of both transgenes. Northern blot analysis was carried out on total cellular RNA isolated from tissues of a limited number of rats of the 21-4H and 21-4L lines. HLA-B27 mRNA was detected with a 350 bp probe from the HLA-B 3′ untranslated region (probe C in Figure 1A), and $h\beta_2m$ mRNA was detected with the same probe used to detect $h\beta_2m$ genomic DNA (probe D in Figure 1B). RT1 class I mRNA was detected with a 447 bp probe from the 3′ untranslated region of the RT1.A gene. Figures 8 and 9 contain results from age- and sex-matched representatives of the 21-4H and 21- 4L lines and a nontransgenic control.

As shown in Figure 8, the distribution and relative abundance of both B27 and $h\beta_2m$ transgene transcripts among

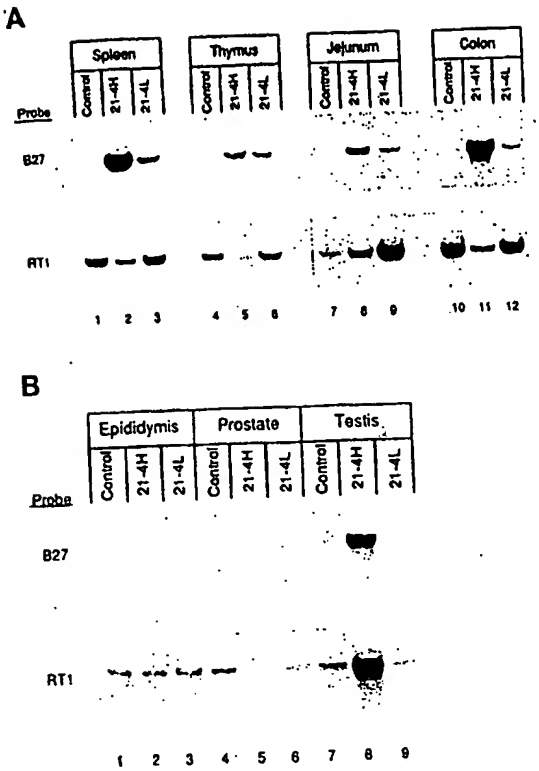

Figure 9. Northern Blot Analysis of HLA-B27 and RT1 mRNA: Comparative Analysis of Seven Tissues

(A) Tissue sources and methods were the same as described in Figure 8. Membranes were exposed 1–8 hr.

(B) Tissue sources were the same as described in Figure 8. Five micrograms of total cellular RNA was added per lane. Prostatic tissue in the 21-4H animal was difficult to identify because of severe atrophy, presumed to be due to loss of androgen stimulation. The membrane probed for B27 was exposed for 1 hr. A 10 hr exposure showed B27 transcripts in 21-4H epididymis (data not shown). The membrane probed for RT1 was exposed for 5 hr.

the various tissues examined were similar to those of the endogenous RT1 class I expression and typical of MHC class I gene expression (Klein, 1986). In addition, both transgenes produced mRNA transcripts of the predicted size.

Figure 9 shows direct comparisons of the 21-4H and 21-4L lines with respect to the relative amounts of B27 and RT1 transcripts in tissues affected by the disease process in the 21-4H line. The abundance of B27 transcripts was dramatically higher in the 21-4H rat than in the 21-4L rat in spleen, colon, and testis, and less markedly increased in jejunum and epididymis. In the thymus, the B27 transcripts were approximately equal in the two lines; however, this may have been a reflection of thymic atrophy in the 21-4H rats.

Although the apparent reduction of RT1 cell surface expression in PBLs was comparable in 21-4H and 21-4L rats (Figure 2C), at the level of mRNA there was no apparent reduction of RT1 transcripts in the 21-4L tissues examined. In contrast, the abundance of RT1 transcripts was markedly reduced in 21-4H spleen, thymus, and colon, compared with tissues from a nontransgenic rat. High expression of RT1 mRNA was found in the 21-4H testis and jejunum. In the case of testis, this probably reflects the intense infiltration of inflammatory cells seen histologically in this organ (Figure 7B), whereas an explanation for the finding in jejunum is less apparent.

Discussion

Integration and Expression of HLA-B27 and $h\beta_2m$ Transgenes in Rats

In an attempt to create an animal model of B27-associated disease, we developed transgenic technology in rats and produced inbred rats expressing both HLA-B27 and $h\beta_2m$. Simultaneously, Mullins et al. (1990), using similar methods, were independently successful in producing transgenic rats expressing a mouse renin gene.

The levels of B27 and $h\beta_2m$ mRNA transcripts in the transgenic tissues paralleled those of the endogenous class I genes in nontransgenic tissues, suggesting that the transgenes were subject to physiologic regulation. It is interesting that the presence of the human transgenes resulted in an apparently reduced expression of the endogenous class I RT1 genes, at the level of cell surface protein expression in PBLs and/or at the level of mRNA, both in lymphoid and nonlymphoid tissue. The possibility was not excluded that the reduced binding of OX18 antibody to the transgenic PBLs was due to an effect of $h\beta_2m$ either on the number of cell surface RT1 class I molecules or on the affinity of the OX18 antibody for these molecules. However, such an effect would not explain the prominent reduction in RT1 mRNA transcripts seen in the 21-4H spleen, thymus, and colon.

Several transcriptional regulatory elements have been identified in the 200 bp 5′ to the transcription initiation site in murine class I MHC genes, including the binding site for the conserved nuclear factor KBF1 (David-Watine et al., 1990; Kieran et al., 1990), and homologous sequences are found in the HLA-B27 promoter region (Weiss et al., 1985). Thus, at least part of the inhibition of RT1 transcription in the 21-4H tissues might be explained by competition by the transgenes for nuclear factor binding.

The Inflammatory Disease of the 21-4H Transgenic Rats: Comparison with B27-Associated Disease in Humans

B27-associated disorders in humans encompass a spectrum of inflammatory diseases affecting predominantly the peripheral and axial musculoskeletal system, gastrointestinal tract, genital tract, integument, and eye (Table 1). Less common involvement of heart and nervous system and rare involvement of lung are also observed in these disorders (Bulkley and Roberts, 1973; Good, 1974; Taurog and Lipsky, 1990). The spontaneously arising disease in B27/$h\beta_2m$ transgenic rats showed a striking clinical and histologic similarity to B27-associated disease in humans, with inflammatory lesions of peripheral and axial joints, gut, male genital tract, nails, skin, and heart. The close resemblance of the findings in the transgenic rats

to B27-associated disease in humans strongly supports the conclusion that the B27 molecule itself participates in the pathogenesis of the various lesions found in different organ systems in the spondyloarthropathies.

The most prevalent site of inflammation in the transgenic rats appears to be the gastrointestinal tract. All of the 21-4H rats under observation for at least 6 months developed overt diarrhea, and a similar picture is emerging in the 33-3 line. These findings suggest that the events initiating the disease process occur in the gastrointestinal tract and that further investigation of the intestinal immunophysiology and immunopathology of the transgenic animals may provide some insight into the role of the B27 molecule in these events.

Numerous observations in humans support a causal link between factors in the gut and inflammatory joint disease. Peripheral and axial arthritis are common accompaniments of chronic inflammatory bowel disease even in the absence of B27 (Table 1), and recent evidence suggests that milder degrees of gastrointestinal inflammation are closely correlated with the occurrence of B27-associated joint disease in individuals without bowel symptoms. Histologic examination of endoscopically obtained biopsies in a large series of patients with reactive arthritis or ankylosing spondylitis indicated that over 60% had asymptomatic inflammatory lesions of the terminal ileum or colon (Cuvelier et al., 1987). Whether patients with B27-associated disease develop inflammatory lesions in the more proximal small intestine or stomach that might resemble those seen in the 21-4H rats is not known.

Although gastrointestinal inflammation in the transgenic rats was present equally in both sexes, arthritis occurred predominantly in males. This closely followed the pattern in humans, in whom males with ankylosing spondylitis, juvenile onset spondyloarthropathy, or reactive arthritis following genital infection outnumber females 3- to 10-fold. The prevalence of subclinical gastrointestinal inflammation in B27 individuals without rheumatic disease, either male or female, is not known. Both peripheral and axial arthritis occurred in the 21-4H rats. Clinically, the peripheral arthritis resembled that seen in other experimental models of arthritis in rats, such as those induced by complete Freund's adjuvant or streptococcal cell walls, with swelling and erythema of the proximal hind paw being the predominant lesion. Histologically, the involved joints showed lesions typical of experimental arthritis in rats, as well as B27-associated peripheral arthritis in humans, with synovial hyperplasia, inflammatory cell infiltration, pannus formation, and destruction of articular cartilage and bone (Greenwald and Diamond, 1988; Taurog et al., 1988b).

Axial arthritis, with inflammatory cell infiltration and periosteal reaction at the margins of the intervertebral discs, was seen histologically in the tails of 21-4H rats. This appears to be the same pathologic process that leads to the vertebral changes in ankylosing spondylitis, although histologic comparison of this lesion with human spondylitis is made difficult by the paucity of descriptions of early lesions in humans (Ball, 1971; Eulderink, 1990). More generally, the vertebral lesion in the 21-4H rats also closely resembles the enthesitis, inflammation at ligamentous attachments to bone, that is a pathologic hallmark of the B27-associated diseases in humans (Ball, 1971).

Dramatic psoriasiform skin and nail lesions developed in the 21-4H rats. These lesions show an extraordinary histologic resemblance to psoriatic lesions in humans. Although in most patients with psoriasis vulgaris there is no association with HLA-B27, lesions termed keratoderma blenorrhagica that are histologically indistinguishable from the psoriatic variant pustular psoriasis are commonly found in B27-associated reactive arthritis (Good, 1974; Keat, 1983). Furthermore, typical psoriasis vulgaris occasionally supervenes in patients initially presenting with reactive arthritis. Finally, a common pathogenetic mechanism between psoriasis vulgaris and B27-associated disease is suggested by the recent observation that both psoriasis vulgaris and the skin lesions of Reiter's syndrome appear to be significantly exacerbated in patients with coexistent infection with the human immunodeficiency virus HIV-1 (Duvic et al., 1987).

Another striking lesion in the 21-4H rats was orchitis, which was found in virtually all of the males, invariably in association with epididymitis. In humans, urogenital inflammation is prevalent in B27-associated diseases. Although urethritis in males with reactive arthritis is a common finding even in the absence of known urethral infection, prostatitis and epididymitis in males, cervicitis in females, and cystitis in both sexes have been described (Yli-Kerttula, 1984). Although there have been no reports of histologically confirmed orchitis associated with HLA-B27 or with B27-associated syndromes, clinical descriptions suggestive of orchitis have been published (Montanaro and Bennett, 1984). It is thus not altogether unlikely that the inflammatory process induced by B27 in the 21-4H rat testis has a milder human counterpart.

Inflammatory disease involving the root of the aortic valve and myocardium was found in the 21-4H rats. Both aortic insufficiency and cardiac conduction disturbances are well-documented complications of ankylosing spondylitis and reactive arthritis (Bergfeldt et al., 1988; Bulkley and Roberts, 1973; Good, 1974). Moreover, primary myocardial disease may also be relatively prevalent in ankylosing spondylitis (Brewerton et al., 1987). The cardiac pathology of the 21-4H rats, like the lesions in the peripheral and axial joints, gastrointestinal tract, skin, and male genital tract, thus appears to be a direct counterpart of a pathologic process in B27-associated human disease.

In comparing the pathologic lesions identified in the B27 transgenic rats with B27-associated disease in humans, only the neurologic disease in the 21-4H LEW line seemed to represent a significant anomaly. Occasional cases of either central or peripheral neurologic disease have been reported in association with B27-associated reactive arthritis (Good, 1974; Montanaro and Bennett, 1984; Taurog and Moore, 1986), but none of these has been characterized histologically, nor do their clinical descriptions resemble the findings in the 21-4H rats. As mentioned under Results, the neurologic lesions in the 21-4H rats appear to be temporally and histologically unrelated to the inflammatory disease seen in other organs.

Although the possibility cannot be excluded that the neurologic disturbance contributed indirectly to the inflammatory lesions, for example by disruption of the normal innervation of lymphoid tissue or gut (Anderson, 1990), the absence of neurologic disease in the 33-3 line, a second transgenic line exhibiting spontaneously occurring B27-associated disease, suggests that the neurologic disease in the 21-4H line is not a necessary part of the inflammatory process in other organ systems, but likely a result of a dominant insertional mutation. A complete description of the neurologic findings in the 21-4H line is in progress.

The Inflammatory Disease of B27/hβ_2m Transgenic Rats: Possible Mechanisms

It is unclear why overt inflammatory disease developed in only two of the seven transgenic rat lines, 21-4H and 33-3. It is unlikely that differences in postconceptional environment play a significant role in determining the phenotypes of the different transgenic lines, since segregation of the diseased phenotype with the 21-4H locus was uniformly observed in litters containing both 21-4H and 21-4L offspring. Insertional mutation appears unlikely as an explanation, since two independent transgenic lines developed aspects of a similar disease. Nor was evidence obtained for differences in B27 function, since the 21-4H and 21-4L lines comparably stimulated immune recognition of B27 by cytolytic T cells. The variation among transgenic rat lines most likely can be ascribed to either quantitative or qualitative differences in the expression of the transgenes or to differing effects of the transgene on the host genome.

The results presented in this study do not exclude the possibility that a human class I MHC gene other than HLA-B27 might also be capable of producing a disease process similar to that described here, nor do they exclude the possibility that the hβ_2m gene alone might be sufficient to produce disease. Studies are in progress to address these possibilities.

Several lines of evidence have suggested that interactions between B27 and bacterial products are involved in the pathogenesis of the spondyloarthropathies (Yu et al., 1989). Although the disease in the transgenic rats arose spontaneously in the apparent absence of infection by pathogens, the possibility must be considered that the pathogenesis involves interactions between B27 and commensal organisms such as the intestinal flora or pathogens not detected by routine serologic screening. Studies in which the transgenic rats are maintained germ free will be important in exploring this issue.

Despite extensive investigation of the structure and function of class I MHC genes in general and HLA-B27 in particular, it has so far not been possible to identify the molecular mechanism of the association of B27 with human disease. However, given the close resemblance of the spontaneous disease of the 21-4H line to B27-associated human disorders, a detailed cellular and molecular analysis of the B27/hβ_2m transgenic rats should enhance our understanding of the role of HLA-B27 in causing disease. It may also contribute to a broader understanding of the function of class I MHC molecules.

Experimental Procedures

Animals

Specific pathogen-free inbred Lewis/CrlBR (LEW) and Fischer F-344/CrlBR (F344) rats, and outbred Sprague-Dawley rats, were purchased from Charles River Laboratories, Boston, MA. Hybrid mice of the transgenic line 56-3 (Taurog et al., 1990), which express high levels of both B27 and hβ_2m on lymphoid cell surfaces, were bred in our animal colony. Animals were maintained in accordance with institutional guidelines.

Generation and Identification of Transgenic Rats

Immature LEW or F344 female rats were superovulated according to the method of Armstrong and Opavsky (1988) and bred with fertile males. The day following breeding, fertilized one-cell eggs were flushed from the oviduct of females exhibiting either vaginal plugs or sperm in vaginal lavage fluid. Eggs were held in Brinster's medium for 2 hr or less before microinjection. Microinjection of eggs and transfer to day 1 pseudopregnant Sprague-Dawley females were carried out essentially as described for mice (Brinster et al., 1985).

Two genomic clones were used for microinjection of fertilized rat eggs (Figure 1). The HLA-B27 gene encoding the HLA-B* 2705 subtype (Bodmer et al., 1990) was contained on a 6.5 kb EcoRI fragment (clone pE.1-B27; Taurog et al., 1988a; Taurog and El-Zaatari, 1988) and the hβ_2m gene was contained on a 15 kb SalI–PvuI fragment (clone pβ2m-13, the gift of Dr. H. L. Ploegh, Amsterdam, The Netherlands; Güssow et al., 1987). Each insert was separated from plasmid DNA by agarose gel electrophoresis and isolated by perchlorate elution (Chen and Thomas, 1980). The solution used for microinjection contained both fragments, each at 1.5 ng/μl.

Identification and quantitation of transgenes were determined in the founder animals and their progeny by dot-blot hybridization of genomic DNA isolated from tail biopsies, as previously described (Brinster et al., 1985). Genomic DNA was analyzed by hybridization with 5′ and 3′ flanking probes for the *HLA-B* locus, as previously described (probes A and B in Figure 1A; Taurog et al., 1988a), and with a 3.7 kb BglII fragment containing exons 2 and 3 of the hβ_2m gene (probe D in Figure 1B).

RNA Analysis by Northern Blot Hybridization

Northern blot hybridization was carried out as described elsewhere (S. D. Malka, L. Laimonis, A. Messing, and R. E. Hammer, submitted). Briefly, total cellular RNA was extracted from tissues by the guanadinium isothiocyanate–CsCl procedure, separated on glyoxal agarose gels, and blotted onto nylon membranes. HLA-B27 mRNA was detected with the 350 bp HLA-B 3′ untranslated region probe pHLA-1.1 (probe C in Figure 1A; Koller et al., 1984), and hβ_2m mRNA was detected with the same 3.7 kb BglII fragment used to detect hβ_2m genomic DNA (probe D in Figure 1B). RT1 class I mRNA was detected with a 447 bp PvuII–HindIII fragment containing the 3′ untranslated region of the RT1.A^a gene pBS3.3/1 (the gift of Dr. J. C. Howard, Cambridge, England; Rada et al., 1990). All stringency washes were carried out in 0.1× SSC, 0.5% SDS at 65°C.

Monoclonal Antibodies, Indirect Immunofluorescence, and Flow Cytometry

The following murine monoclonal antibodies were used: B.1.23.2, IgG_{2b}, binding a monomorphic determinant shared by HLA-B and -C molecules (Rebai and Malissen, 1983); BBM.1, IgG_{2b}, binding hβ_2m (Brodsky et al., 1979); and OX18, IgG_1, binding a monomorphic rat RT1 class I antigen (Fukumoto et al., 1982). P1.17, an IgG_{2a} myeloma, served as a negative control.

Indirect immunofluorescence was carried out as previously described (Taurog and El-Zaatari, 1988; Taurog et al., 1988a). Briefly, Ficoll–Hypaque-purified peripheral blood mononuclear cells were incubated with saturating concentrations of each monoclonal antibody, washed, then incubated with fluorescein-conjugated $F(ab)'_2$ fragments of goat anti-mouse Fcγ antibodies (Cappel Inc., Malvern, PA). After washing, the cells were fixed in 1% paraformaldehyde before analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Viable lymphocytes were analyzed by gating of forward and 90° light scatter.

Generation and Analysis of Cytolytic T Cells

Primary alloimmunization by skin grafting was carried out by the method of Peter and Feldman (1972). Seven days after graft placement, recipient spleen cells were used as effector cells in a 4–6 hr ^{51}Cr release assay, as previously described (Taurog et al., 1988a). Two mouse L cell lines were used as target cells, one transfected with and expressing the $h\beta_2m$ gene, the other transfected with and expressing both the HLA-B*2705 and $h\beta_2m$ genes, as previously described (El-Zaatari et al., 1990).

Histology

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Joints were embedded and sectioned following fixation and decalcification for 4–6 weeks in 10% disodium EDTA, as previously described (Taurog et al., 1988b), or following decalcification in 10% formic acid. Eyes were embedded in methacrylate before sectioning and staining.

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